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Research Note

Characterization of Diarrheagenic *Escherichia coli* and *Salmonella enterica* from Produce in the Chobe District of Botswana

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ABSTRACT

Diarrheal disease is a leading cause of death in children in low- and moderate-income countries. Fresh produce, including fruits and vegetables, may harbor diarrheal disease-causing bacteria including strains of *Salmonella enterica* and *Escherichia coli*. This study aimed to determine the prevalence and antibiotic resistance profiles of *S. enterica* and *E. coli* isolated from produce samples (n = 207) obtained from retail markets in northern Botswana in Chobe District of Botswana in 2022. Samples were enriched in the appropriate selective media: Brilliant Green Bile Broth for *E. coli* and Rappaport Vassiliadis Broth for *S. enterica*. *E. coli* were confirmed by PCR detecting the *phoA* gene, and classified as potentially pathogenic through screening for the *eae*, *stx, and stx2* and *estIb* genes. *S. enterica* isolates were confirmed using *invA* primers. Isolates were evaluated for resistance to ampicillin, amoxicillin-clavulanic acid, chloramphenicol, cefotaxime, doxycycline, streptomycin, sulfamethoxazole, and tetracycline antibiotic using the Kirby-Bauer Disk Diffusion method. *E. coli* was isolated from 15.5% of produce samples (n = 207). The gene *eae* was detected from 1.5% of samples, while *stx1, stx2,* and *estIb* were not detected. Resistance to one or more antibiotics was common (72%) with the majority of the resistant *E. coli* (n = 32) isolated from fruits (22%) and greens (18%) compared to other types of vegetables. Multidrug resistance (MDR, resistant to 3 or more antibiotics) was identified in 18% of samples. *S. enterica* was isolated from 3.4% of produce samples (7, n = 207). Resistance was uncommon among the *S. enterica* isolates (1/7). Overall prevalence of diarrheagenic *S. enterica* and *E. coli* was low; however, their presence and that of MDR *E. coli* in foods commonly consumed raw increases the risk to vulnerable populations. Strategies to reduce contamination of fresh produce and public education on washing and cooking some types of produce may be useful to reduce disease.

Diarrheal disease is an ongoing threat that continues to negatively impact the lives of those all around the world, especially in low-and moderate-income nations (LMIN) in Sub-Saharan Africa including Botswana (Boshi-Pinto et al., 2006). In Africa, attribution data for diarrheal disease are incomplete, but it is estimated that two groups of bacterial pathogens (Diarrheagenic *Escherichia coli* and *Salmonella enterica*) are major contributors (Msolo et al., 2020). In LMIN, diarrheagenic strains of *E. coli* have been reported among the leading causes of morbidity and mortality among children under the age of five (Tanih et al., 2014.) In Botswana, nontyphoidal serotypes of *S. enterica* are estimated to be one of the chief causes of death in its HIV-endemic population (Rowe et al., 2010). Bacteria, including diarrheal pathogens, may acquire resistance to commonly used antibiotics, which could promote treatment failures. Antibiotics are easily obtained in LMIN and may be taken without the identification of etiological agent (Alexander K. pers. com.). It is estimated that by 2050, antibiotic-resistant bacterial (ABR) infections will be responsible for 10 million deaths annually across the globe (Chandra et al., 2013). There is growing concern that ABR resistance is spreading at a faster rate in the Sub-Sahara because there are few agencies monitoring its emergence (Bedekelabou et al., 2020).

Fresh fruits and vegetables are part of a healthy diet; however, they are also implicated in *E. coli* and *S. enterica* outbreaks, including the circulation of ABR strains (Holzel et al., 2018). These pathogens and ABR can contaminate food or food-derived products at many points in preharvest or postharvest by contact with fecal waste during farming, in contaminated irrigation or wash water, or in soils used for fertilizer (Rahman et al., 2022). Contaminated produce may serve as a

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vehicle, transporting pathogens and ABR to other regions where it is consumed (Abakpa et al., 2018, Berg et al., 2014). While limited prevalence data for *S. enterica* and pathogenic *E. coli* is available for the Chobe District of Botswana, prevalence data in neighboring areas show it is established in the region. The Chobe District in northern Botswana is semi-arid and home to a diverse array of herbivore wildlife, notably including one of Africa's largest populations of elephants. As a result, commercial agriculture in the region is minimal. Much of the Chobe region's food is imported from other areas in Botswana as well as other neighboring countries such as South Africa. Variants of diarrheagenic *E. coli* have been isolated in South Africa including enteroinvasive (EIEC) from irrigated lettuce, enterotoxigenic (ETEC) from pig farms (Ogundare et al., 2018), and enteropathogenic *E. coli* (EPEC) in 19.2% (32/170) of water isolates (Bolukaoto et al., 2021). Prevalence of *E. coli* and *S. enterica* in foods available for sale in the Chobe region of Botswana may more closely resemble that of foods in other regions and serve as a source for pathogens and ABR.

This study aimed to characterize potential virulence and antibiotic resistance of *E. coli* and nontyphoidal serotypes of *S. enterica* isolates isolated from produce that are commonly consumed raw purchased from retail markets in the Chobe Region of Botswana. These foods may serve as a source of pathogens and ABR that may impact the health of humans and wildlife in a pristine environment without commercial agriculture. Identifying sources of harmful bacteria can allow for appropriate policy development and education to improve health and build strong local economies.

Materials and methods

Produce sample collection and processing in Botswana

Vegetables were obtained from three different markets (formal and informal) in Kasane, Botswana. Depending on the availability the purchases included two or more samples of fruits (apples, pears), greens (spinach, collard greens, rape, cabbage, and lettuce), above ground vegetables (tomato, and yellow, green, and red peppers), below ground vegetables (carrots), and soil associated vegetables (cucumber, celery, butternut squash, and pumpkin). Samples purchased were packaged in bags or on trays wrapped with plastic wrap. Samples were collected each month during 2022. All produce samples screened were free from visible mold or decay. All samples were processed within 24 h of collection. After processing, produce samples were frozen and shipped to Virginia Tech for further processing.

A. Produce sample preparation and enrichment

Produce samples were prepared in three ways. The intact fruits and vegetables were placed into a sterile plastic bag with enough 0.1% peptone to submerge or float the sample. A sterile peeler was used to completely remove the outermost peel from cucumbers, squash, and carrots. The peels were placed into a sterile whirlpack bag. For head-forming leafy greens, the outermost leaves were obtained, cut into 25 g pieces, and placed in a sterile filter bag. The processed produce samples then had 50 mL of 0.1% sterile peptone (Becton, Dickinson and Company, Sparks, Maryland) added into the filter bags Once processed as outlined above, all produce samples were placed on the orbital shaker (Oxford Lab Products, San Diego, California) for 10 min at 2,000 rpm.

The peptone rinse from the processed samples was decanted into two 15 mL falcon tubes and centrifuged (Axiology Labs, Gauteng, South Africa) at 3,000 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in 13 mL of the appropriate selective media: Brilliant Green Bile Broth (BGBB) (Becton, Dickinson and Company) for *E. coli*, and Rappaport Vassiliadis Broth (RV) (Becton, Dickinson and Company) for *Salmonella*. The BGBB and RV were incubated at 42 °C for 4 h and then at 37 °C for 20 h. After incubation, the produce enrichments were centrifuged for 10 min at 2000 rpm to pellet the cells. The pellet was resuspended in 250 μL of sterile 0.1% peptone and dispensed into two 1.5 mL culture tubes with Tryptic Soy Broth (Becton, Dickinson and Company) with 20% glycerol and sent to Virginia Tech for bacterial isolation.

B. Bacterial isolation and DNA extraction

The enriched vegetable pellets received from Botswana were plated on Eosin Methylene Blue Agar (EMB, Becton, Dickinson and Company) and XLD (Becton, Dickinson and Company) and incubated at 37 °C for 24 h. Characteristic green sheen colonies (EMB) and black colonies (XLD) were selected and restreaked for isolation a minimum of four times. DNA was isolated from a single putative *E. coli* colony following the manufacturer's instructions for the Biobasic EZ-10 Spin Column Soil DNA Mini-Preps Kit (Biobasic, Amherst, NY). DNA was isolated from putative *Salmonella* isolates by heating an isolated colony suspended in 100 μL of nuclease-free water for 30 s in a PCR tube within a thermacycler on the boil cycle.

PCR detection of E. coli genes

A multiplex PCR was used to simultaneously confirm the identification of *E. coli* using the *phoA* gene (Kong et al., 1999), and detect genes associated with virulence: *eae* designed to detect Enterohemorrhagic and Enteropathogenic pathotypes, and *estIb* to detect *Enterotoxigenic E. coli* (Vidal et al., 2005; Müller et al., 2007). The sizes of the expected mPCR products and concentrations of the primer pairs are listed in Table 1. The mPCR amplification was conducted in a total reaction volume of 25 μL containing 12.5 μL Gotaq Green Master Mix (Promega, Wisconsin, USA) 1 μL Magnesium chloride 25 μM (Promega), 7.5 μL Nuclease Free Water, 2 μL of DNA template, and the corresponding primers (Table 1). Nuclease-free water was used for the negative control. DNA from Enterohemorrhagic *E. coli* O157:H7 ATCC 43895 (ATCC) and Enterotoxigenic *E. coli* H10407 (Serotype O78:H11) NR-4 (BEI Resources, NIAID, NIH, USA) were used for positive controls. The mPCR conditions were 95 °C for 5 min. followed by 28 cycles of 95 °C for 30 sec., 62 °C for 30 sec., 72 °C for 30 sec., and a final extension at 72 °C for 5 min.

PCR was also used for the detection of Enterohemorrhagic *E. coli* using the *stx1* and *stx2* genes. This PCR was performed when an *E. coli* isolate contained the *eae* gene. The sizes of the expected PCR products and the concentration of the primer pairs are listed in Table 1. The mPCR amplification was conducted in a total reaction volume of 25 μL containing 12.5 μL Gotaq Green Master Mix 2X, 10.5 μL Nuclease Free Water, 1 μL of DNA template, and the corresponding primers. Nuclease-free water was used for the negative control. DNA from Enterohemorrhagic *E. coli* O157:H7 ATCC 43895 was used as a positive control. The PCR conditions were 94 °C for 5 min. followed by 35 cycles of 94 °C for 30 sec., 62 °C for 30 sec., 72 °C for 1 min., and a final extension at 72 °C for 5 min. The PCR products were electrophoresed on 2% agarose gel using 1x Tris-acetate-EDTA buffer stained with ethidium bromide. The gel was visualized under a UV transilluminator. Bands of corresponding size (Table 1) were excised and prepared for sequencing using the Zymoclean Gel DNA Recovery Kit (Zymo) before Sanger DNA sequencing was performed to confirm the sequence.

PCR detection of Salmonella

Primer sets for *invA* were used for the confirmation of *Salmonella* characteristic black colonies on XLD using previously established methods. PCR amplification was conducted in a total reaction volume of 25 μL containing 12.5 μL Gotaq Green Master Mix, 10.5 μL Nuclease Free Water, 2 μL of cellular extract, and at 0.4 μM *invA* primers

Table 1
The *E. coli* primers used and their corresponding attributes

Gene	Sequence (5′–3′)	Size (bp)	Target pathotype	Concentration	Reference
eae - F	TCAATGCAGTTCCGTTATCAGTT	482	*EHEC*	0.4 μM	Vidal et al. (2005)
eae - R	GTAAAGTCCGTTACCCCAACCTG			0.4 μM	
phoA - F	GTGACAAAAGCCCGGACACCA TAAATGCCT	903	*E. coli control*	0.24 μM	Kong et al. (1999)
phoA - R	TACACTGTCATTACGTTGCGGATTTGGCGT		*E. coli control*	0.24 μM	
estIb - F	TGTCTTTTTCACCTTTCGCTC	171	*ETEC*	0.2 μM	Chandra et al. (2013)
estIb - R	CGGTACAAGCAGGATTACAACAC			0.2 μM	
stx1 - F	CGATGTTACCGTTTGTTACTGTCACAG	216	*EHEC*	0.4 μM	Müller et al. (2007)
stx1 - R	AATGCCACGCTTCCCAGAATT			0.4 μM	
stx2 - F	GTTTTGACCATCTTCGTCTGATTATTGAG	343	*EHEC*	0.4 μM	Müller et al. (2007)
stx2 - R	AGCGTAAGGCTTCTGCTGTGAC			0.4 μM	

(Malorny et al., 2003). Nuclease-free water was used for the negative control. DNA from *Salmonella enterica* ser. Tennessee K4643 was used as a positive control. The PCR conditions were 96 °C for 2 min. followed by 35 cycles of 96 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products were electrophoresed on 2% agarose gel using 1x Tris-acetate-EDTA buffer stained with ethidium bromide. The gel was visualized under a UV transilluminator. A Band of the corresponding size was excised and prepared for sequencing using a gel extraction kit before DNA sequencing.

Serotyping of Salmonella

Four of the isolates identified as having the *invA* gene were sent for serotyping at the United State Department of Agriculture's National Veterinary Services Laboratory.

Antibiotic resistance testing

PCR confirmed *E. coli* and *S. enterica* isolates from food were tested for susceptibility to 12 different antibiotics using the disk diffusion method on Mueller-Hinton Agar according to the guidelines of the Clinical Laboratory Standard Institute (CLSI, 2023). The antibiotics and concentration in each disk were amoxicillin-clavulanic acid 20/10 μg (AMC), ampicillin 10 μg (AM), azithromycin 15 μg (AZI), chloramphenicol 30 μg (CHL), cefotaxime (CTX), ciprofloxacin 5 μg (CIP), doxycycline 30 μg (DOX), gentamicin 10 μg (GEN), meropenem 10 μg (MER), streptomycin 10 μg (STR), tetracycline 30 μg (TET), and trimethoprim/sulfamethoxazole (SXT). Inhibition zone diameters around the antibiotic-impregnated disks were measured in mm and rounded to the closest integer before being compared to the CLSI clinical breakpoints to classify each bacterial isolate as resistant, intermediate, or susceptible. Only isolates that were confirmed as *E. coli* by PCR amplification of the *phoA* gene or *Salmonella* by PCR amplification of the *invA* gene were tested. Multiclass resistance was defined as resistance to three or more classes of antibiotics.

Statistical analysis

All statistics were performed in R: A Language and Environment for Statistical Computing (V4.1.2; R Core Team). The following packages were utilized: "tidyverse" and "readxl" to upload the data, "knitr" to view the data. The data were not normally distributed requiring non-parametric tests. A Fisher's Exact test was used to determine if there was a significant association between bacteria (*S. enterica* or *E.coli*) presence and source attributes including: vendor/source, season, quarter, and food group. Since the availability of different fruits and vegetables varied across the months, it was necessary to create broader sample categories for analysis. The Fruit Group contains apples and pears. The Greens group contains spinach, collard greens, rape, cabbage, and lettuce. The Above Ground group includes tomato, and yellow, green, and red peppers. The Below Ground group included carrots. The Soil Associated group contains cucumber, celery, butternut squash, and pumpkin. Relationships were considered significantly dependent at $P \leq 0.05$.

Results

E. coli was isolated from 15.5% of produce samples (32/207). The sample type was significantly associated with *E. coli* isolation, with *E. coli* most often isolated from greens ($p = 0.001$) (Fig. 1). The gene *eae* was detected from 1.45% (3/207) of samples, while *stx1, stx2,* and *estIb* were not detected. The *eae* positive samples came from apples, carrots, and cucumbers collected from the same vendor in the same month. There was no statistical association between the incidence of *phoA* and the vendor (results not shown). *E. coli* isolation from produce was not impacted by the time of the year during which samples were collected (quarter, $P = 0.97$) (Fig. 2) or the rainfall season ($P = 0.96$, results not shown). Resistance to ampicillin, amoxicillin-clavulanic acid, chloramphenicol, cefotaxime, doxycycline, streptomycin, sulfamethoxazole, and tetracycline was seen in *E. coli* isolates from 72% (23/32) of produce samples (Table 2). *E. coli* isolates resistant to one antibiotic were identified from 25% (8/32) of samples. Resistance to two antibiotics was seen in *E. coli* isolates from 9.3% (3/32) of samples. Multi drug resistant (3 or more) isolates were obtained from 18.7% (6/32). The *E. coli* isolates from produce were most often resistant to ampicillin and tetracycline (Table 2). No isolates were resistant to azithromycin, gentamycin, or meropenem. MDR *E. coli* were more frequently isolated from greens (collards, rape, lettuce) and fruit (apples and pears) ($P = 0.01$). Comparable numbers of MDR were present in produce collected during wet and dry seasons (results not shown).

S. enterica were isolated from 3.4% of produce samples (7/207) belonging to the fruits, above ground, and below ground associated categories. Isolates from fruit (3) and the above ground category (2) were identified as *Salmonella enterica* serotype Enteritidis, but isolates from the below ground group could not be serotyped. *S. enterica* isolation from produce occurred only in samples collected from July to September ($P < 0.001$). Each of the *S. enterica* isolates was susceptible to ampicillin, amoxicillin, azithromycin, ciprofloxacin, cefotaxime, doxycycline, gentamicin, meropenem, streptomycin, trimethoprim/sulfamethoxazole, and tetracycline. One isolate was resistant to chloramphenicol.

Discussion

E. coli is widely used as an indicator species for fecal contamination in food and water sources (Odonkor & Ampofo, 2013). This study saw the most *E. coli* isolations came from fruits and vegetables that are often eaten raw, therefore increasing the chance of transmission to humans, domestic animals, or wildlife that may consume these

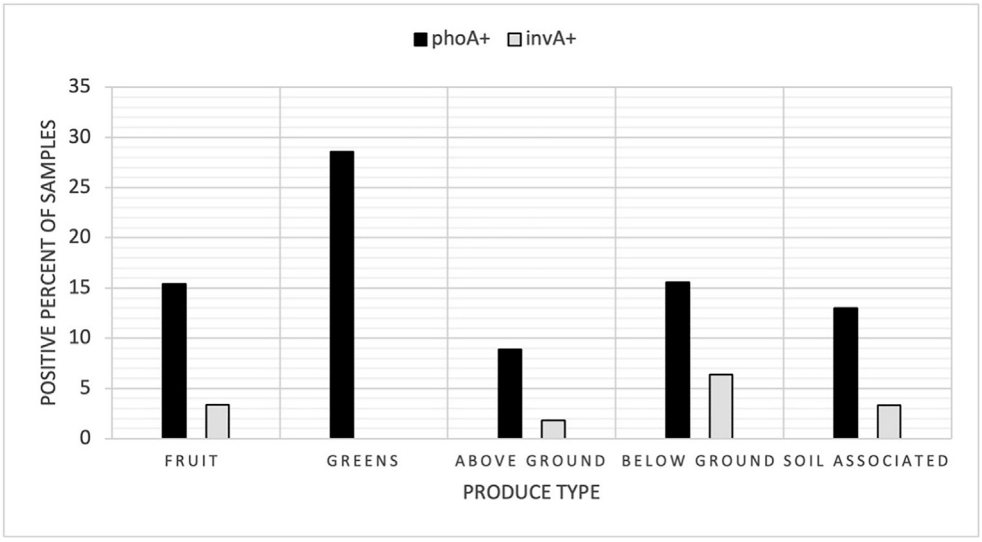

Figure 1. Percent of produce samples containing the *phoA* and *invA* gene from different food types collected from the Chobe Region of Botswana, 2022. Fruit (N = 71), Greens (N = 28), Above Ground (N = 45), Below Ground (N = 32), Soil Associated (N = 31). No *invA* genes were detected in greens.

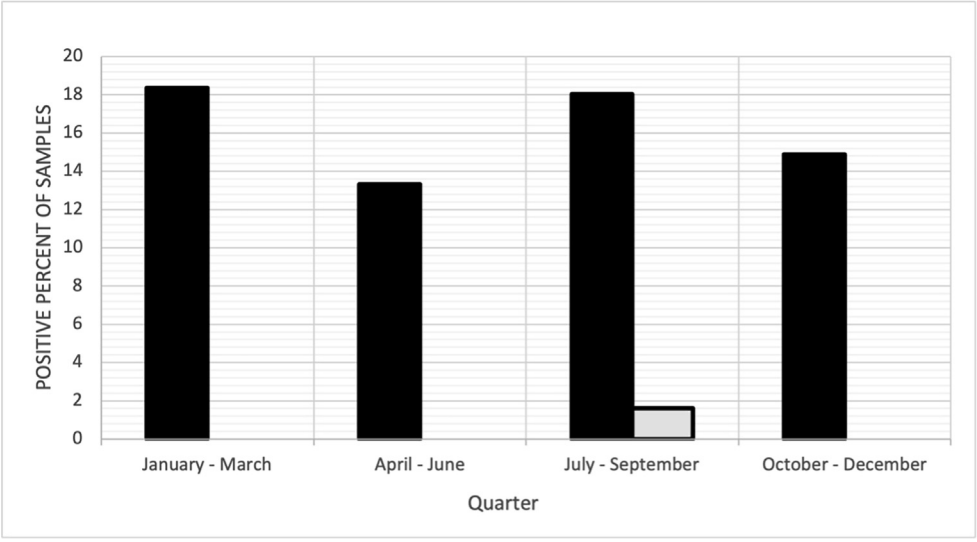

Figure 2. Percent of produce samples from the Chobe Region of Botswana, 2022 containing the *phoA* and *invA* gene collected during different time periods.

Table 2
Total samples with *E. coli* isolates resistant to each antibiotic/antibiotic class tested by Food Type from isolates collected monthly from Botswana, 2022.

Food Group	AMC	AM	AZM	C	CTX	D	GM	MEM	S	SXT	TE	MDR
Fruit	1	2	0	1	1	3	0	0	2	0	2	3
Greens	0	1	0	0	0	3	0	0	3	1	6	3
Above Ground	0	0	0	0	0	0	0	0	0	0	0	0
Below Ground	0	3	0	0	0	0	0	0	0	0	0	0
Soil Associated	0	2	0	0	0	0	0	0	0	0	0	0

AM – Ampicillin/Penicillin class: AMC – Amoxicillin and clavulanic acid/Penicillin and beta-lactam classes: AZM – Azithromycin/Macrolide class: C – Chloramphenicol/Chloramphenicol class: CTX – Cefotaxime/beta-lactam class: D – Doxycycline/Tetracycline class: GM – Gentamycin/Aminoglycoside class: MEM – Meropenem/Carbapenum class: S – Streptomycin//Aminoglycoside: SXT – Trimethoprim/Sulfamethoxazole/Sulfonamides: TE – Tetracycline/Tetracycline class, MDR- Resistant to 3 or more drugs.

products. Isolates from pears, apples, lettuce, spinach, carrots, and cucumbers contained the *eae* gene, but were negative for *stx1, stx2,* therefore, it is unclear if they are definitely pathogenic. The *eae* gene is present in many diarrheagenic *E. coli*, in particular the enteropathogenic and enterohemorrhagic pathotypes (Hamner et al., 2019). The locations of the farms where these items were grown are unclear; however, some products including apples and pears were labeled as grown in South Africa. Diarrheagenic *E. coli* have been isolated from soils, water, and locally produced greens in South Africa (Iwu et al., 2021, Richter et al., 2022). Low prevalence of *eae,* has also been reported in *E. coli* isolates from spinach, lettuce, parsley, carrots, cucumber, and tomatoes (Özpınar et al., 2013, Waturangi et al., 2019). There

was no association between seasonality and the isolation of *E. coli* from produce. This may be explained because much of the produce available in grocery stores is grown in other regions of Botswana or in South Africa where differences in water sources used for irrigation, organic soil amendments, and animal intrusion may impact distribution patterns (Williams et al., 2015).

Many serotypes of *Salmonella enterica* are associated with human illnesses and may be transmitted through unhygienic conditions or contaminated water. Produce samples containing *Salmonella* came from two different vendors during quarter three (July-September). The *Salmonella* were isolated from pears, carrots, green peppers, cucumbers, and apples. Isolation of *Salmonella* from fruits and vegetables is rarely reported in Africa (Al-Rifai et al., 2020). The levels in this study are lower than reported in other studies from India of 27.5% (11/140) in spinach, 6.2% (5/80) in cucumbers, and 3.7% (3/80) in carrots (Verma et al., 2018). Previous studies have suggested a seasonality association between *Salmonella* detection in produce from Chile, with higher detection taking place in spring and summer (Toro et al., 2022).

Fresh produce, especially when consumed raw, is a source of exposure to antibiotic-resistant bacteria and genes of clinical importance (Rahman et al., 2022). Contamination of produce occurs through the same mechanisms; however, the types of antibiotic resistance may be influenced by antibiotics administered to livestock or humans in the area. Resistance to antibiotic drugs available over the counter has been noted in pathogenic *E. coli* in Southern Africa (Van et al., 2020). In this study, *E. coli* resistance was highest to ampicillin and tetracycline, two classes of antibiotics commonly administered to cattle, poultry, and pigs in Africa (Kimera et al., 2020). The majority of the ampicillin *E. coli* isolates were not resistant to amoxicillin, suggesting there is limited transmission of plasmids transmitting extended beta-lactamase resistance (Galindo-Méndez, 2020). *E. coli* isolates with resistance to ampicillin, amoxicillin, azithromycin, ceftriaxone, chloramphenicol, gentamycin, streptomycin, trimethoprim-sulfamethoxazole, and tetracycline have been reported from produce in South Africa (Jongman & Korsten, 2016; Du Plessis et al., 2017). This highlights that the fresh produce available for consumption in the area may introduce pathogens and ABR from its growing region.

Antibiotic resistance monitoring programs in the Sub-Saharan Africa are limited and typically focus on food from animal origin. Increased surveillance of ABR in humans, animals, and the environment is important to create and support implementation of meaningful policies that seek to minimize spread. Mechanisms to reduce preharvest contamination with ABR and pathogens can include improved manure disposal, treatment of manure before soil application, and improving the quality of irrigation water. Postharvest contamination may also occur especially through the application of contaminated water used to wash or spritz the produce. Inclusion of sanitizers, wrapping, and waxing of the produce (as appropriate) may be strategies that further reduce postharvest contamination. Future studies should compare the types of resistance from human-isolated *E. coli* and their genetic relationships to determine the contributions of fresh produce to distribution of pathogens and antibiotic resistance in the Kasane, Botswana area. Targeting education programs that focus on reducing contamination such as washing produce or cooking (greens such as rape, collard greens, and kale) and improving hygiene behaviors that may reduce the spread is crucial.

CRediT authorship contribution statement

Auja Bywater: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Galaletsang Dintwe:** Methodology, Investigation, Data curation. **Kathleen A. Alexander:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Monica A. Ponder:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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