Characterization of *Campylobacter sp.*, *Salmonella enterica,* and Diarrheagenic *Escherichia coli* From Food, Food Waste, and Water in the Chobe Region of Botswana

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Scientific Abstract

Introduction and Justification: Diarrheal disease is a leading cause of death in children in low- and moderate-income countries. Food, food waste, and water are all vehicles that can promote the spread of diarrheal disease-causing bacteria like *Campylobacter, Salmonella enterica,* and *E. coli*. Resistance to commonly used antibiotics is on the rise, making them difficult to manage. This study aimed to determine prevalence and antibiotic resistance profiles of *Campylobacter, S. enterica,* and *E. coli* isolated from food, food waste, and water samples obtained from the Chobe Region of Botswana. In addition, the survival of two common pathogens, *E. coli* and *C. jejuni,* on kale, a type of leafy green commonly consumed raw, was determined.

Methods: Samples were collected from the Chobe region of Botswana in 2022 including water from the local river, food (produce, beef, pork, and poultry) from local vendors, and food scraps from the landfill. Food samples were enriched in the appropriate selective media: Brilliant Green Bile Broth for *E. coli*, Bolton Broth for *Campylobacter*, and Rappaport Vassiliadis Broth for *S. enterica*. Water samples were collected using modified USEPA methods1103.1 and 1604, E.coli isolation was performed by plating on RAPID *E.coli*2 agar and incubation at 37°C for 2h and 44°C for 16-22h. *Campylobacter, S. enterica,* and *E. coli* were isolated from meat, poultry, and water samples before being sent to Virginia Tech, while enriched bacterial pellets from the produce were shipped for screening and isolation at Virginia Tech. *E.coli* were confirmed by PCR detecting the *phoA* gene (all *E. coli*), and classified as pathogenic through screening for the

eae (present in enterohemorrhagic and enteropathogenic *E.coli*), *stx1 and stx2* (present in enterohemorrhagic *E. coli)* and *est1b* (present in Enterotoxigenic *E.coli)* genes. *Campylobacter* isolates were confirmed using a genera-specific PCR while *S. enterica* isolates were confirmed using *invA* primers. These enrichment and primer sets were tested as part of a study to determine the survival of *E. coli* O157:H7 and *C. jejuni* on kale during a 21-day shelf life. *E. coli* and *S. enterica* isolates were subjected to antibiotic resistance testing using the Kirby-Bauer Disk Diffusion method.

Results: Methods for detection of inoculated *E. coli* O157:H7 on kale indicated survival for the majority of the shelf-life (up to 19 d), in comparison, *C. jejuni* was undetectable by day 13 using enrichment and PCR or plating. From the Botswanan samples, *E. coli* was isolated from 20% of produce, 49% of meat, and 84.7% of water. *Salmonella* was only isolated from produce samples (2.4%, 7/294). Resistance was uncommon among the *Salmonella* isolates with only one isolate being resistant to chloramphenicol. No *Campylobacter* were isolated from the screened produce, meat, or food waste. *E. coli* resistant to 3 or more classes of antibiotics (MCR) were identified in 15.5% of produce, and 22.2% of meat isolates. Isolation of *E. coli* or *Salmonella* from meat was not associated with a particular food type. In contrast, isolation of *E. coli* was more common from certain types of vegetables and fruits. Antibiotic-resistant *E. coli* were isolated more commonly from beef, poultry, and pork than from produce. Multi-class resistant *E. coli* were isolated from fruits, greens, soil associated, and above ground associated vegetables, beef, and poultry. Water samples were collected from the same time period as the food samples. *E. coli* isolation, especially pathogens (based on *eae* presence) was more frequent from environmental water samples collected during the wet season compared to the dry season. Water samples collected during periods of increased rainfall were more likely to contain *E. coli* isolates,

especially pathogens. *S. enterica* and Diarrheagenic *E. coli* isolates, especially MCR isolates, pose a significant risk of illness to consumers. Strategies to reduce the circulation of these pathogens in foods and water sources are needed.

Characterization of *Campylobacter, Salmonella,* and Diarrheagenic *Escherichia coli* From Food, Food Waste, and Water in the Chobe Region of Botswana

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General Audience Abstract

People can get sick with diarrheal diseases after consuming contaminated food and water. These illnesses are difficult to treat and control when the bacteria causing them are resistant to antibiotics. *Campylobacter, Salmonella,* and diarrheagenic *E. coli* are three types of bacteria that can cause illness from food and water. These illnesses disproportionately affect people, especially children, in low-and moderate-income countries like Botswana. Little is known about the prevalence of *Campylobacter, Salmonella,* and diarrheagenic *Escherichia coli* in Botswana. This study aimed to determine the prevalence of these microorganisms as well as how resistant they are to different types of antibiotics. Samples from produce, beef, poultry, pork, and recreational water sources were collected in the Chobe region of Botswana over the course of 2022. Food samples were collected from different vendors and food scraps were obtained from the landfill. *E. coli* and *Salmonella* were isolated out of meat and water samples in Botswana while produce samples were shipped as mixed cultures to Virginia Tech where *E. coli, Salmonella,* and *Campylobacter* isolation or confirmation was done. Once the target bacteria were isolated, their resistance to certain antibiotics was tested. *Salmonella* was only found in produce from samples collected during October-December. No *Campylobacter* was found from produce, meat, or food waste. More *E. coli* was isolated from fruit or vegetable food waste collected from the landfill than from produce bought at local vendors. *E. coli* was obtained from meat purchased from local vendors more often than samples from the landfill. *E. coli* was found more often in water when there was more rainfall. *E. coli* that causes illness was also more likely to be obtained during the wet seasons. Resistant *E. coli* that could not be killed by the

screened antibiotics, were classified as multi-drug resistant when it was resistant to more than three antibiotics. Rainfall, season, and the food source influenced if *E. coli* isolates were likely to be multi-drug resistant. While more research is needed to determine how these bacteria are moving in the environment and gaining resistance to antibiotics, the findings of this study show they are present in the environment and require further research.

Attributions

- This research could not have been completed without the dedicated efforts of the Alexander laboratory in Kasane, Botswana. Galaletsang Makgarebe Dintwe prepared the food samples for enrichment and isolated *E. coli* and *Salmonella* from meat samples, Lefang Chobolo screened all meat samples for *Campylobacter*, and Letty Lekone isolated *E. coli* from water samples. Thanks to all the other members of the lab that have assisted with other necessary tasks to upload the data and shipped the samples to Virginia Tech.
- Thank you to the co-authors of Chapter 3 in this thesis.
    - Kathleen Alexander: writing – review & editing, supervision, funding acquisition, and conceptualization
    - Joseph Eifert: Writing – review & editing, conceptualization
    - Laura K. Strawn: Writing – review & editing, software, resources, data curation, conceptualization
    - Monica A. Ponder: Writing – review & editing, Writing – original draft, supervision, methodology, investigation, funding acquisition, and conceptualization
- Thank you to the undergraduates that contributed to the antibiotic resistance testing: Rachel Saunders, Evan Nuckolls, Christine Kelly, and Madison Fleming.
- The Fralin Life Sciences Institute at Virginia Tech performed Sanger sequencing on PCR products to confirm the specificity of PCR performed as confirmation in this thesis.

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Table of Contents

Introduction and Justification

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 Africa. While the true yearly burden of diarrheal diseases is unknown, an estimated 30 million people suffered from severe diarrhea in Africa with a resulting 330,000 deaths in 2015 alone (Reiner et al., 2018). These diarrheal illnesses contribute to a majority of deaths of young African children (Nguyen et al., 2021). Diarrheal disease is a complex issue that is influenced by many different factors in the environment. Diarrheal diseases may be caused by infection from microorganisms, chiefly viruses, bacteria, and parasites. Transmission of these illnesses may be person to person, or through ingestion of foods or water that have been contaminated. In Africa, attribution data for diarrheal disease is incomplete but it is estimated that three groups of bacterial pathogens (*Campylobacter spp.*, *Salmonella enterica,* and Diarrheagenic *E. coli*) are major contributors. *Campylobacter spp.* are widespread across Africa with *Campylobacter jejuni* being the most frequently attributed to disease (Thomas et al., 2020). Data about *Campylobacter* prevalence in Africa is incomplete, but suggests it is endemic with a 10-year study finding *C. jejuni* and *C. coli* in 21% (415/1,941) of children sampled in Malawi. *Campylobacter*-associated diarrhea in children under the age of five is estimated at 2% in Sudan and as high as 21% in South Africa (Asuming-Bediako et al., 2019; Kaakoush et al., 2015). A meta-analysis looking at seven African countries reported *S. enterica* prevalence at 19.9% in raw foods and 21.7% in ready-to-eat foods (Paudyal et al., 2017). In LMIN, diarrheagenic strains of *Escherichia coli* have been reported among the leading causes of morbidity (illness) and mortality (death) among children under the age of five (Tanih et al., 2014). A molecular study reported an estimation that upwards of 80% of children in South Africa had been infected by enteroaggregative *Escherichia coli* at

some point in their lives (Aijuka et al., 2018). Transmission of these pathogens through food, food waste, and water may be impacted by seasonality, as well as the extent and sources of fecal contamination in the environment.

Food plays an important role in the transmission of diarrheal diseases. Globally, countries in Africa south of the Sahara have one of the highest incidence and death rates resulting from foodborne illness (Jaffee & Grace, 2020). *Campylobacter spp.*, *S. enterica*, and Diarrheagenic *E. coli* are three potentially pathogenic bacteria that can be found in food. *Salmonella* and *E. coli* have been frequently reported on meats, fruits, and vegetables (Duffy et al., 2005). Both *Campylobacter* and *Salmonella* were found in animal meat and organ samples obtained in Botswana (Thomas et al., 2020). *Campylobacter* was found in 37.7% of 11,828 poultry samples, 24.6% of 1,975 pig samples, 17.8% of 2,907 goat samples, 12.6% of 2,382 sheep samples, and 12.3% of 6,545 cattle samples. *Salmonella* from the study was found in 13.9% of 25,430 poultry samples, 13.1% of 5,467 pig samples, 5.3% of 72,292 cattle samples, 4.8% of 11,335 sheep samples, and 3.4% of 4,904 goat samples. A 1992 outbreak in southern Africa identified consuming beef as a risk for becoming sick with *E. coli* O157:NM (Effler et al., 2001). The literature shows the potential for these microorganisms to be present in different types of foods and the environments where they are processed.

Transmission through water contaminated with fecal matter is important to consider when trying to understand diarrheal disease. Water sources can act as a vehicle for pathogenic microorganisms that cause diarrheal disease. Diarrheal illnesses in Botswana are cyclical, with peaks in March and October during the wet and dry seasons, respectively (Alexander et al., 2013). There is a strong correlation between these peaks and climatic conditions, including environmental surface water levels. Pathogenic *E. coli* from various sources, including human

feces can contaminate the soil (Pickering et al., 2012). Soil and associated microbes can be transported via water from rainfall overland where they accumulate in larger water sources that people may use, potentially resulting in increased illness.

The spread of antibiotic-resistant (ABR) bacteria, including *E. coli*, *S. enterica*, and *Campylobacter spp.* are a global public health threat. Antibiotic-resistant strains of bacteria can be found in food, animals, humans, and the environment (Vikesland et al., 2017). Certain populations in African nations have shown an increase in *E. coli* resistance to many commonly used antibiotics (Ekwanzala et al., 2018). It's well known that human activities contribute to increased rates of ABR through the use of antibiotics for disease treatment, but ABR is also increased through improper disposal of antibiotics and their excretion in the urine where antibiotics may leach into the environment and water sources (Larsson & Flach, 2022). However, it's not only *E.coli* isolated from humans or livestock that may develop resistance to antibiotics. *E. coli* isolates from 150 different wild African animals collected from the Chobe region of Botswana showed a 13.3% multidrug resistance rate (Jobbins & Alexander, 2015). These were not animals that routinely had antibiotics administered to them, indicating these bacteria were acquired from their environment. ABR incidence among isolates from food animals, fish, and vegetables is increasing (Founou et al., 2016). There has been a clear increase in resistance genes among pathogenic bacteria from many sources in the environment. What is less clear is how that resistance is being passed.

This project is part of a grant that aims to develop tools and knowledge to advance basic scientific understanding, theory, and modeling tool development for diarrheal diseases. There are complex couplings driven by the movement of agricultural products, associated food waste, and scavenging wildlife with a focus on ABR and foodborne pathogen movement in the Chobe

region of Botswana. This project fulfills the grant by striving to characterize *E. coli*, *S. enterica*, and *Campylobacter spp.* isolated from surface water, foods (beef, poultry, pork, and produce), and food wastes collected in the Chobe region of Botswana. The region in this study is located at the edge of the Chobe National Park in Botswana and the prevalence of *Campylobacter*, *Salmonella*, and *E. coli* in foods consumed in this area is unknown. It has a large population of wildlife and limited agricultural production locally meaning food is sourced from other regions of Botswana, Zimbabwe, and South Africa. These influences make it an interesting place to look at certain diarrheal pathogens and their corresponding spread of ABR. The project used traditional culturing methods for isolation of *E. coli*, *S. enterica*, and *Campylobacter spp.* and multiplex PCR protocols to determine pathogenicity for different virulence genes present in diarrheagenic *E. coli*. The antibiotic resistance profiles of the isolates were compared using the Kirby-Bauer disk diffusion method. This project will help characterize the role of surface water, food, and food wastes in the transmission of *Campylobacter*, *Salmonella*, and diarrheagenic *E. coli* disease and antibiotic resistance in the region, as well as set a foundation for further genomic research.

Objectives

1. Molecular characterization of isolates from the Chobe Research Institute in Botswana will be performed using multiplex PCR (mPCR). Two different mPCR protocols were necessary: 1) *Campylobacter* mPCR for discrimination between *C. coli* and *C. jejuni* species 2). *Escherichia coli* mPCR protocol was optimized to screen for pathogenicity genes present in Enterohemorrhagic, Enteropathogenic, and Enterotoxigenic *Escherichia coli*. The efficacy and limit of detection were determined from pure cultures and from a model system of kale. In addition, the survival of *Campylobacter jejuni* and *E. coli* O157:H7 on kale was determined over

a 21-d shelf life using both classical microbiological and the mPCR methods. These findings are outlined further in Chapter 3.

2. Characterize putative isolates of *Escherichia coli, Salmonella,* and *Campylobacter* from foods, water, food waste, and human fecal samples from Botswana for classification in the target genus. The protocols were refined to accurately test all samples. Meat, produce, water, and human fecal samples were collected in Botswana. In Botswana, the *Escherichia coli, Salmonella,* and *Campylobacter* were isolated from water, meat and human fecal samples and sent to Virginia Tech for further characterization using mPCR and antibiotic susceptibility testing. All vegetable pellets were processed in Botswana and sent to Virginia Tech for isolation of *E. coli and S. enterica.* These findings are further outlined in Chapter 4.

$H1_o$: The type of food will not impact the prevalence of *E. coli, Salmonella, or Campylobacter* found.

$H1_a$: The type of food will impact the prevalence of *E. coli, Salmonella, or Campylobacter* found.

$H2_o$: The store the food samples were taken from will not impact the prevalence of *E. coli, Salmonella,* or *Campylobacter* found.

$H2_a$: The store the food samples were taken from will impact the prevalence of *E. coli, Salmonella,* or *Campylobacter* found.

$H3_o$: The rainfall in the Chobe region will not impact the prevalence of *E. coli, Salmonella,* or *Campylobacter* found in food, food waste, water, or human fecal samples.

$H3_a$: The rainfall in the Chobe region will impact the prevalence of *E. coli*, *Salmonella*, or *Campylobacter* found in food, food waste, water, or human fecal samples.

3. Characterize isolates for potential pathogenicity through PCR and antibiotic resistance using the Kirby-Bauer disk diffusion method. This is further outlined in chapter four.

   a. All isolates that were confirmed as belonging to *S. enterica*, *Campylobacter spp.*, and *E. coli* were tested using the Kirby-Bauer disk diffusion method following the guidelines of the Clinical Laboratory Standard Institute (CLSI, 2023).
   b. All pathogenic *E. coli*, *Salmonella*, and *Campylobacter* isolates were pathotyped through PCR primers. Identified *E. coli* pathotypes were compared among sample types to look for potential links.

$H1_o$: Isolated *Campylobacter*, *Salmonella*, and *E. coli* from poultry collected during this study will not have a statistically significant higher rate of multidrug resistance (resistant to 3 or more antibiotic classes) than isolates from produce.

$H1_a$: *S. enterica*, *Campylobacter*, and *E. coli* from isolates from poultry collected during this study will have a statistically higher rate of multidrug resistance (resistant to 3 or more antibiotic classes) than isolates from produce.

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Chapter 2:
Review of Literature

Background on *Campylobacter, Salmonella,* and diarrheagenic *Escherichia coli*

Foodborne illness is a global threat. *Campylobacter*, *Salmonella*, and diarrheagenic *Escherichia coli* are three prominent foodborne bacteria that contribute to illness, decreased quality of life, and even death. These bacteria can spread through consumption of contaminated food, person-to-person, and water. These pathogens disproportionally affect the young, elder, and immunocompromised resulting in higher numbers of reported cases and deaths (Gourama, 2020). The burden of these pathogens is discussed in Chapter 1.

Campylobacter sp. are Gram-negative bacteria that have an S-shaped, or spiral morphology. This bacteria can be found in animals, water, and the environment. *Campylobacter jejuni* and *coli* are two species most commonly isolated from animals that result in human illness (Rukambile et al., 2019). *Campylobacter* is often found in birds, poultry, and environments where poultry are raised and slaughtered (Tegtmeyer et al., 2021). *Campylobacter* spp. are prevalent in nature and colonize the gastrointestinal tract of warm-blooded animals (cattle, sheep, pigs, and poultry) typically without causing symptoms (Lazou et al., 2021). *Campylobacter jejuni* grows best at a temperature from 37°C to 42°C which makes birds an ideal reservoir with a body temperature of 41°C to 42°C (Vandamme et al., 2015). The environment, human, and animal matrix present unique challenges that must be overcome by *Campylobacter* in order for it to survive and colonize in organisms. The biggest challenges are overcoming exposure to oxygen, non-ideal temperatures for growth, desiccation, and various stress factors (Murphy et al., 2006). Food handling practices and sanitation also impacts the spread and survival of

Campylobacter sp. Despite the numerous challenges *Campylobacter* must overcome, it is still persistent and problematic in food which warrants studying it further.

Escherichia coli are mostly Gram-negative bacilli found in the gut of mammals, water, and the environment. Most strains of *E. coli* are typically harmless to humans and may be part of the human microbiome. *E. coli* species are introduced to the environment via fecal contamination where it becomes a functional part of soil and water microbial communities (Ishii & Sadowsky, 2008). However, some *E. coli* strains are pathogenic. These strains are referred to as diarrheagenic *E. coli* and result in a variety of gastrointestinal symptoms. These strains are classified as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (EAEC) dependent on genes associated with disease and symptoms. Serotypes within the EHEC, known as the big 6, are naturally found in the intestinal tracts of ruminant animals including cattle, goats, sheep, deer, and elk but do not result in illness (Kolodziejek et al., 2022). However, in humans, the *eae* gene allows for attachment and a variety of other acquired genes including *stx1* and *stx2* which promote illness (Denamur et al., 2021). *E. coli* species are able to reproduce in temperatures from 7°C to 50°C but have an ideal growth temperature of 37°C (Fotadar et al., 2005). *E. coli* may be restricted by the nutrients and energy sources available to it. *E. coli* can overcome this in the environment through metabolizing their cellular carbohydrates, alternating catabolic functions, and increasing catabolic flexibility (Van Elsas et al., 2011). As a last resort, *E. coli* can enter a dormant state to facilitate survival when exposed to low nutrients and low temperatures (Oliver, 2010). *E. coli* has a plethora of mechanics to survive in the environment which keeps it at the forefront of food safety focus.

Salmonella enterica is Gram-negative and rod-shaped bacteria. It is found in animals, water, and the environment. *S. enterica* are further classified into serotypes, of which there are 2,463, based on biochemical and antigenic identification (Brenner et al., 2000). *S. enterica.* can also be classified into two groupings based on host preference and disease manifestation, typhoidal and non-typhoidal. *S. enterica* infections in humans come from both typhoidal and non-typhoidal serovars (Gal-Mor et al., 2014). *S. enterica* serotypes can be found throughout the environment as well as the intestinal tracts of domestic and wild animal species, especially poultry, cattle, pork, rodents, and reptiles (Wiedemann et al., 2015). Food contaminated with *S. enterica* can further spread growth and contamination through inadequate storage temperatures, cross-contamination, and insufficient cooking temperatures (Carrasco et al., 2012). *S. enterica can* grow from 8°C to 54°C but has an ideal growth temperature of 35°C -37°C (Podolak et al., 2010). The different survival mechanisms used by *Salmonella* are constantly studied and evaluated to improve risk mitigation.

Prevalence of *Campylobacter, S. enterica,* and Diarrheagenic *E. coli* in the Sub-Sahara

Illnesses due to *Campylobacter, S. enterica,* and diarrheagenic *E. coli* infection are a global concern, but looking in localized regions can provide useful information to combat these threats. Each geographic region is unique and thus it is difficult to apply broad global data to a smaller area and maintain a tailored plan to alleviate local challenges. One challenge is obtaining representative data in areas without a lot of testing or where under-reporting is common. Despite this, it is still useful to understand the larger area to better understand how to employ efforts and future research. The information below attempts to focus on Botswana and includes other countries in Sub-Sahara Africa.

Globally, diarrhea continues to be a top cause of death in children under five resulting in 1.6 million deaths annually (Manetu et al., 2021). It is estimated that 14% of diarrheal diseases in Botswanan children are the result of *Campylobacter spp.* (De Vries et al., 2018). Chicken, red meat, pasteurized milk, and contaminated drinking water are all important vehicles for *Campylobacter* to infect humans (De Vries et al., 2018). In addition to food, *Campylobacter* can also be found in feces and the environment. *Campylobacter* in poultry has been reported in Africa with ranges from 2% to 90% in chicken carcasses (Asuming-Bediako et al., 2019). Regionality influences incidence, so the study also looked at specific countries in Africa. In Kenya, chicken has a 77% (77/100) positivity rate while beef is lower at 2% (1/50). South Africa has a positivity rate in chicken of 32.3% (32/99). A study looking at water as a source of illness in South Africa found an overall *Campylobacter* recovery rate of 21.7% (20/92) with the highest prevalence being in well or harvested rainwater stored in a container (Chukwu et al., 2019). While studies in surrounding countries can be helpful, more research is needed to fully understand the bigger picture of the prevalence and burden of disease *in* Botswana.

A study from 2003-2008 conducted by the Botswana National Health Laboratory found that *Salmonella* and *Shigella* were the most common bacterial pathogens causing diarrheal illness (Samaxa et al., 2012). Out of 221 fecal samples taken from children in Botswana under 5 with diarrhea, 8 were positive for *Salmonella* (Urio et al., 2001). *S. enterica* is found in poultry, beef, various fruits and vegetables, human feces, and the environment. Across 27 African countries, *S. enterica* was detected in 13.9% (3,534/25,430) of poultry samples, 13.1% (716/5,467) of pig samples, 5.3% (3,831/72,292) of cattle samples and 3.4% (167/4,904) of goat samples (Thomas et al., 2020). This study found that external samples, such as hides and feathers, were more likely to be contaminated than meat and organ samples. This is a concern for areas, like those often

found in Africa, where hygienic slaughtering with proper food safety protocols may not be in place, allowing for cross-contamination from animal hides to food processing and meat surfaces. One study conducted in South Africa found 1,944 *Salmonella enterica ser. Enteritidis* isolated from farm animals (614), meat (832), animal feed (3), environment (461), and other sources (33) showing that *S. enterica* may be present in multiple locations on a farm (Magwedere et al., 2015). Two abattoirs were sampled in Botswana and *Salmonella* was present in 28% (A) and 23% (B) of the environmental samples collected (Motsoela et al., 2002). Abattoir A had 50% (22/44) *Salmonella* presence in soils from corrals, 50% (26/52) in stunning areas, 40% (4/10) in feces, 30% (8/26.7) in knives, 30% (2/6.7) in saw blades, and 50% (8/16) in water. Abattoir B had 50% (21/42) in soils from corrals, 50% (17/34) in stunning areas, 40% (5/12.5) in feces, 30% (3/10) in knife blades and saw blades, and 50% (9/18) in water.

EPEC, ETEC, EIEC, EHEC, EAEC, and DAEC have all been associated with diarrheal disease in regions of Africa (Okeke, 2009). ETEC prevalence from pig farms in South Africa was reported at 78.8% (26/33), 70% (119/170), and 52% (13/25) in large, medium, and small-scale pig farms, respectively (Ogundare et al., 2018). An EAEC and DAEC gene was found in 8% (4/48) and 4% (2/48) irrigated water isolates from South Africa, respectively (Aijuka et al., 2018). The same study reported 6% (2/29) of irrigated lettuce isolates containing genes common to EIEC. EPEC was reported in 19.2% (32/170) of water isolates taken from South Africa (Bolukaoto et al., 2021). EHEC *E. coli* O157:H7 has been reported infrequently in South Africa among humans (0.77, 1/220), water (2.3%, 3/220), and cattle and beef (27.7%, 36/220) (Ateba & Mbewe, 2011). A study in Gaborone, Botswana found EHEC O157:H7 prevalence in 5.22% of meat cubes (7/134), 3.76% of minced meat (5/133), and 2.26% of fresh sausages (3/133) (Lupindu, 2018). Proximity to meat-producing animal facilities can increase the risk of higher

prevalence of *E. coli* in water as found in a study conducted in the Northwest province of South Africa (Ateba & Mbewe, 2011). *E. coli* prevalence in water is dependent on several environmental factors. For example, wet and dry seasons with their associated rainfall have been shown to correlate with diarrheal disease outbreaks in the Chobe district of Botswana with *E. coli* concentrations being higher during the wet season (Fox & Alexander, 2015). Most relevant data in the Sub-Sahara is limited to *E. coli* O157:H7. This highlights a need for more in-depth research into different species and countries.

Transmission and Survival in the Environment

When looking at the role of food in transmission, *Campylobacter, S. enterica,* and diarrheagenic *E. coli* are all transmitted in similar ways. One form of transmission is through the contamination of food or water with contaminated fecal matter from animal hosts (Thomas et al., 2020). Additionally, people may become ill through contact with live animals and environments that have become contaminated with the animal host feces. Transmission can also occur through vectors, such as the common housefly, indirectly spreading the pathogen from the contaminated feces to a food or water source (Nichols, 2005). Data is limited for Africa, but other regions of the world can provide context on the transmission of these biological agents. Over the course of 17 years, 143 *Campylobacter sp.* outbreaks were studied in England, Wales, and the United Kingdom (Kaakoush et al., 2015). Contaminated food or water is the transmission source responsible for 114 outbreaks, animal contact for two, and 22 with unknown modes of transmission. In the United States, 484 *Salmonella* outbreaks from 2009-2014 were identified; 99 were attributed to animal contact and 385 to foodborne transmission (Marus et al., 2019). From 2010-2017, 466 STEC outbreaks were identified, with 361 having known transmissions (Tack et al., 2021). Of those 361 outbreaks, 200 were attributed to foodborne, 87 to person-to-person, 49

to animal contact, 20 to water, and 5 to environmental contamination. The variety of transmission routes creates a complex picture of disease transmission in an environment.

Food and waterborne transmissions are likely to be the largest sources for transmission in Sub-Saharan Africa. Hygienic conditions, sanitation, and access to resources like clean water and safe food are necessary to reduce pathogen transmission. Greater than 70% of people in Eastern and Southern Africa don't have access to basic sanitation services (UNICEF, n.d.). UNICEF also reports that of this population, 19% practice open defecation. This is a key factor to consider, especially since *Campylobacter, S. enterica,* and diarrheagenic *E. coli* can be transmitted via the fecal-oral route. Access to wastewater treatment facilities reduces the number of diseases by preventing contamination of water and foods. Living conditions and hygienic resources are crucial considerations when studying the rates of diarrheal illness in the Sub-Sahara.

Survival of these bacteria after transmission allows them to persist in the environment and potentially cause human illness. *Campylobacter* can form a 'viable but nonculturable' state which allows it to persist in food, water, and soil when subjected to low nutrients and unideal survival conditions (Murphy et al., 2006). *Campylobacter* employs numerous tactics such as forming biofilms, gaining micronutrients from neighboring microorganisms, and acquiring essential nutrition from amoeba (Indikova et al., 2015). A study looking at swine production and slaughter found that *C. coli* was able to persist in both pigs and the environment, even with different practices aimed at reducing them (Quintana-Hayashi & Thakur, 2012). *E. coli* can produce filamentous structures which extend from the surface of the cell and aid in attachment to surfaces (Van Elsas et al., 2011). This mechanism aids in the transmission and persistence of the pathogen among soil and food, water and plants, and other forms of food processing and packaging. One study found that *E. coli* O157:H7 persisted in fallow soils for 25-41 days, rye

roots for 47-96 days, and legumes for 25-40 days (Gagliardi & Karns, 2002). *Salmonella* has evolved and acquired tools through horizontal gene transfer like flagella, fimbrial adhesins, pathogenicity islands, LPS modifications, and virulence genes (Staes et al., 2019). These mutations allow for better persistence in food, water, and the environment and subsequent transmission to humans. Once contaminated, *Salmonella* has been shown to persist in calf units from four months to two years, and in empty poultry buildings for at least one year (Rose et al., 2000). Transmission is important in understanding how a micro-organism is introduced into an area, but understanding the micro-organism's survival is also important in preventing illness and further spread.

Transmission and cross-contamination of *Campylobacter, S. enterica,* and diarrheagenic *E. coli* in meat and RTE foods are reduced through proper food handling and hygiene; therefore, it is necessary to look at the behaviors of the people handling food in the Sub-Sahara. Unfortunately, this data is not readily available for this region. One study was conducted that looked at the knowledge of street food vendors in South Africa. It found that 89% (278/312) of participants reported learning about food preparation through observation only, almost 25% (78/312) thought food safety training wasn't necessary for street food vendors, 20% (62/312) were unaware that food handlers can carry microbes, and nearly 84% (262/312) believed using leftover food from the previous day is safe (Marutha & Chelule, 2020). The study also reported that only 40% (125/312) of individuals reported washing their hands with soap and water, 60% (187/312) used the same utensils to serve different foods, and 45% (140/312) used the same water to wash food ingredients and utensils. This study also made a few observations including that nearly 57% (178/312) of vending sites weren't protected from animals or pests, nearly 74% (231/312) did not have adequate hand washing facilities, nearly 58% (180/312) of the time food

was not stored or displayed in sealed containers, and utensils were only cleaned with soap 48.5% (151/312) of the time. These behaviors are all practices that can reduce risk in foodborne transmission and contamination that may result in illness. Although this data is for food vendors only, it is concerning since this population should be more aware of safe food handling than the general population due to training and regulations. Poor practices by food handlers have the potential to result in more illnesses in the event of contamination. More than one of the data points from the study indicates an environment where disease could spread easily among the food. Additionally, a lack of key infrastructure, as illustrated in this study, is a hurdle for increased food safety in many countries. Without proper prevention in place, these pathogens are able to spread easily and cause illness.

Isolation and Identification Methods for *E. coli* and *Salmonella*

Determining the presence of *E. coli* and *Salmonella* from environmental samples requires different methods including culturing and PCR. Traditional culturing methods are used widely across the world. *E. coli* is present in low levels in environmental samples, requiring enrichment in different selective medias like Brilliant Green-bile broth and lauryl sulfate broth and then plating on Eosin methylene blue (United States, Food and Drug Administration, Division of Microbiology, 1984). *Salmonella* enrichment using selective and differential medias like Rappaport-Vassiliadis and xylose lysine desoxycholate agar is necessary for isolation from many different foods (Rasamsetti et al., 2021). One study looking at *S. enterica* serovar Typhimurium and *E. coli* in soil found that both bacteria could only be detected on arugula after enrichment, highlighting their importance in preventing false negatives (Natvig et al., 2002). In addition to traditional culturing methods, molecular methods are also advancing and becoming more commonplace to determine microbial presence often based on presence of pathogenicity-

associated genes. These methods include tests like polymerase chain reaction (PCR), nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP), and quantitative polymerase chain reaction (qPCR) (Fratamico et al., 2016). Other methods such as pulse field gel electrophoresis, MLST, and whole genome sequencing (WGS) are needed to compare isolates to each other (Aly et al., 2019).

Traditional culturing and molecular methods are different, and each brings strengths to bacterial isolation and identification. Both traditional culturing and molecular methods have been used and tested thoroughly. They are reliable and widely accepted across the world as accurate and acceptable approaches. These methods are typically more cost-effective as well providing LMIC countries access to self-sufficiency and sustainability through in-country testing. However, culturing techniques are slow compared to molecular approaches. A strength of molecular testing is that it can be done in as little as a few hours. A study looking at Egyptian foods found that molecular and traditional methods gave the same identification, but the molecular testing was done in 48h compared to the 6d for traditional methods (El-Hadedy & Abu El-Nour, 2012). This could be due to the amount of bacteria present. Another study reported a limit of detection in traditional methods in produce at 1 log CFU/g compared to 0.27-067 log CFU/g for PCR when enrichment was first performed (Bywater et al., 2023). Molecular testing also has the potential to be significantly more sensitive than traditional methods (Azzari et al., 2008). An increased sensitivity would help detect infections or bacteria that may have otherwise gone undetected. Molecular methods can also allow direct screening for pathogenic genes without direct isolation and culturing. However, molecular testing is more expensive than traditional culturing methods. This is a limiting factor for those LMIC countries. Additionally, more training is required to run these tests; an additional resource LMIC countries may not have

access to. Both forms of isolation and identification have pros and cons requiring consideration of the circumstances in which they are being used.

Antibiotic-Resistant Bacteria

Antibiotic resistance is a growing global public health threat. It is estimated that by 2050, antibiotic-resistant infections will be responsible for 10 million deaths annually across the globe (Chokshi et al., 2019). Antibiotic-resistant bacteria (ABR) can contaminate food or food-derived products at many points in pre-harvest or post-harvest, and consumption of pathogenic ABR may result in illness (Founou et al., 2016). The drugs ampicillin, streptomycin, co-trimoxazole, chloramphenicol, and tetracycline saw an increase in resistant bacteria from 31% in 1994 to 42% in 2003 (Kariuki et al., 2005). The increase in resistance was greater than 10% in a 10-year period indicating a large growth in ABR bacteria and a concerning trend. "*E. coli* strains have been reported to be resistant to major classes of antibiotics such as β-lactams, quinolones, aminoglycosides, sulfonamides, fosfomycin and unfortunately, this resistance has spread to last resource antibiotic classes such as the polymyxins and carbapenems" (Galindo-Méndez, 2020). Both *E. coli* and *Salmonella sp.* may acquire genes through mutations or horizontal gene transfer of resistance determinants. These genes can be shared among bacteria through microbial strategies like transposons, integrins, plasmids, and horizontal gene transfer (Wright, 2010). *E. coli* mechanisms of resistance include direct inactivation of antibiotics (B-lactamases), modification of cellular targets (sul1, sul2, aadA), and efflux pumps (tetA, tetB, tetC) (Boerlin et al., 2005). *S. enterica* mechanisms of resistance include gene regulation (*tet(X4*), *mcr-1, mcr-3.1, mcr-5.1, mcr-9, mar),* beta-lactamase, and efflux pump alterations (Grossman, 2016; Lyu et al., 2021). Out of all the world regions, the Sub-Sahara has the least antimicrobial surveillance strategies suggesting ABR may increase more in this area (Bedekelabou et al., 2021). This

highlights the increasing threat of antibiotic resistance in the Sub-Sahara and shows the need for further research to combat this issue.

Food can act as a vehicle for antibiotic-resistant bacteria and their corresponding genes (Caniça et al., 2019). Increased usage of antibiotics in food animal production has facilitated antibiotic-resistant bacteria growth and development in farm environments (Berg et al., 2014). From 2010-2020, multidrug resistance in *S. enterica* isolates from poultry was reported at an average of 12.1% (25/206) in Zimbabwe and 20% (88/440) in Zambia (Ramtahal et al., 2022). Discharge of antibiotic residues and resistant bacteria through fecal waste can result in spread to the soil or dispersal into water where they may come into contact with produce. *E. coli* and *Salmonella* isolates from produce, especially lettuce, have been reported as resistant to antibiotics (Rahman et al., 2021). This same study reported that in the Czech Republic, 86% (13/15) of *E. coli* isolates taken from 108 raw vegetable samples were positive antibiotic-resistant genes including *qac, sul1, tetA, int, sul3, mer,* and *tetB.* This demonstrates that antibiotic bacteria and their genes are prevalent in the food supply and are an imminent public health threat.

Antibiotic resistance traits can be introduced to bacteria in many different places in the environment. It is thought that animal populations play a big part in harboring bacteria that move these antibiotic-resistant traits within other bacteria. A study in Botswana found that 43.4% (62/143) of *E. coli* isolated from wildlife feces exhibited resistance to one or more antibiotics. Further, 13.9% (19/143) of these *E. coli* were identified as resistant to three or more antibiotics, classifying them as multi-drug resistant (Jobbins & Alexander, 2015). This same study found no antibiotic resistance in *E. coli* samples collected from a limited number of domestic cattle populations. This illustrates the variability of resistance profiles in *E. coli* in Botswana. Another

study looking at *Campylobacter* in pork production in South Africa found multidrug resistance in 87.3% (330/378) of the isolates tested (Sithole et al., 2021). This raises an interesting question of how this resistance is finding its way into animal populations. One theory is that this spread could have come from water, natural bacterial population evolution, and overuse of antibiotics.

The fate of bacteria in human feces must also be considered when addressing antibiotic resistance. Depending on the area, this waste can go into wastewater treatment facilities, latrines, or even directly into environmental soil or water sources. Wastewater treatment plants have been known to serve as hotspots for antibiotic resistance and corresponding horizontal gene transfer (Karkman et al., 2019). Looking at individual patients can help give more information on these bacteria in the ecosystem, however, waste-water treatment is also helpful when trying to get a screenshot of the population health (Driver et al., 2022). When open defecation is practiced, indirect person-to-person transmission of ARG's is also a possibility through environmental sources. Widespread littering and unregulated waste, including septic sludge waste, dumping are serious environmental threats in Botswana and the estimated waste that is produced by Botswana greatly exceeds its capacity to properly clean it up (Kgosiesele & Zhaohui, 2010). This allows microorganisms to spread unchecked throughout the environment where they can transfer resistance genes and increase antibiotic resistance in an environment. These micro-organisms can get into the natural water sources and food where they spread further to animals and potable water.

Water is another important factor to look into when studying the spread of antibiotic resistance in an environment. A study was conducted looking for antibiotic resistance among *Salmonella* and *E. coli* in groundwater collected from the Mafikeng area of South Africa (Philemon Thabo Phokela, 2011). The study found that all 64 *Salmonella* isolates obtained had

resistance to ampicillin and vancomycin while 91% (58/64) had resistance to erythromycin. For *E. coli*, 51-78% (32-50/63) of the isolates had resistance to ampicillin and erythromycin at one of the two study sites. This compares to another study site in the North-West Province of South Africa where *E. coli* isolates had an 80-100% (184-230/230) resistance to chloramphenicol, tetracycline, and erythromycin (Kinge et al., 2010). Another study in the Northwest Province of South Africa found that 95% (244/257) of *Campylobacter* isolates identified had resistance to at least one antibiotic with the highest prevalence of resistance to clarithromycin (Chukwu et al., 2019). There is a clear presence of antibiotic resistance among bacteria isolated from water in Africa. This is a concern since water spreads throughout the environment, animals, and human habitats.

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Chapter 3:
Survival of Inoculated *Campylobacter jejuni* and *Escherichia coli* O157:H7 on Kale During Refrigerated Storage

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Abstract

Campylobacter and pathogenic Escherichia coli illnesses have been attributed to the consumption of fresh produce. The leafy green, kale, is increasingly consumed raw. In comparison to other leafy greens, kale has a longer shelf-life. Due to the extended shelf-life of kale, it is warranted to examine the survival of pathogenic *Campylobacter jejuni* and *E. coli* O157:H7 inoculated on the surface of kale stored in a controlled environment at 4 ± 1.4°C, and average humidity of 95 ± 1.9% over a 23-day period. At predetermined time points (days 0, 1, 2, 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21), inoculated kale was destructively sampled and the surviving bacteria determined by serial dilution and plating onto Tryptic soy agar, Charcoal cefoperozone deoxycholate agar, and Eosin methylene blue for total aerobic bacteria, C. jejuni, and E. coli O157:H7, respectively. Enrichment and PCR were used for detection when pathogens were not detected using serial dilution and plating. Aerobic heterotrophic bacteria increased over the 23-day period, in contrast, significant declines in the inoculated pathogens were observed. Inoculated *E. coli* O157:H7 survived longer on kale (up to 19 d); in comparison, *C. jejuni* was undetectable by day 13 using enrichment and PCR or plating. In conclusion, *C. jejuni* and *E. coli* O157:H7 declined on fresh kale over time when held at refrigerated temperatures but were still detected during the majority of the time when the kale would likely still be considered edible by consumers.

Introduction

Raw kale is becoming increasingly popular among consumers due to its nutritional benefits and is commonly eaten in salads or included in beverages (Kang & Song, 2017). Studies have shown that raw kale contains more nutritional content than cooked or heavily processed (Sikora & Bodziarczyk, 2012). However, without a cooking step, pathogenic bacteria have a greater chance of persisting on fresh vegetables for consumption (Luna-Guevara et al., 2019). Leafy greens are associated with the most produce-associated illnesses in the United States: from 2018 to 2021, there were eleven multistate outbreaks investigated by the US Centers for Disease Control and Prevention that were linked to leafy greens (Centers for Disease Control and Prevention, 2022; The Interagency Food Safety Analytics Collaboration, 2021). Serotypes of pathogenic *Escherichia coli* are associated with the majority of leafy green-borne outbreaks in the United States and Canada: from 2009 to 2018, 40 outbreaks were identified with a resulting 1,212 illnesses (Marshall et al., 2020). *Campylobacter* spp. are an emerging concern on fresh produce. From 2004 to 2012, there have been seven outbreaks of *Campylobacter* spp. from fresh vegetable consumption, and those are only the ones that have been reported/detected (Mohammadpour et al., 2018). There are several places along the supply chain that leafy greens, such as kale, can become contaminated with *E. coli* and *Campylobacte*r spp. This contamination may occur where the vegetables are grown, during harvest, and after harvest (Abay et al., 2022). Pathogens are not easily removed by washing produce due to their adhesion capabilities (Berger et al., 2010). *E. coli* O157: H7 can survive for extended periods at 4°C storage; for example, 12 d on iceberg lettuce (Delaquis, Bach, & Dinu, 2007) and 15 d on baby spinach (Lopez-Velasco et al., 2010).

Among leafy greens, kale has a longer shelf-life, often exceeding 15 d, when stored at temperatures below 5°C and high relative humidity (Albornoz & Cantwell, 2016). Wilting and loss of chlorophyll (discoloration) are associated with major losses to kale product quality, but high numbers of naturally occurring microorganisms (aerobic bacteria, *Enterobacteriaceae, Pseudomonas* spp., and yeast and mold) may lead to flavor, appearance, and structure changes during storage (Ragaert et al., 2007). There are few studies that have characterized the changes to kale microbial communities during extended storage (Mansur & Oh, 2016), especially in the presence of human pathogens (Mansur & Oh, 2015). Some background microorganisms are reported to antagonize the growth of *E. coli* O157:H7 at permissive temperatures on produce (Lopez-Velasco et al., 2012), but may also alter the survival at refrigerated temperatures. The purpose of this study was to evaluate the survival of *C. jejuni* and *E. coli* O157:H7 inoculated separately onto freshly harvested kale, bagged, and stored under refrigeration until the kale appearance indicated it was beyond acceptable shelf-life, as indicated by exudate, yellowing or wilting. In addition, total aerobic plate counts were performed along the time course, which may aid in the prediction of product shelf-life.

Materials and Methods

Bacterial Cultivation and Inoculum Preparation

All cultures were maintained at −80°C in 20% glycerol stock. *C. jejuni* ATCC 33291 was inoculated into Bolton Broth (ThermoFisher Scientific, Waltham, MA) with supplement (SR0155E) without blood and incubated at 37°C for 48 h in 10% CO_2 in a MIDI 40 CO_2 incubator (Thermo-Fisher Scientific). *E. coli* O157:H7 strains, ATCC 43894 and ATCC 43895, were streaked for isolation from freezer stocks onto Levine EMB Agar (ThermoFisher Scientific) and incubated for 24 h at 37°C. Individual colonies were then taken from the plate and

transferred to Tryptic Soy Broth (Becton, Dickinson and Company, Sparks, Maryland) and incubated at 37°C for 24 h. The cultures were centrifuged at 2817g for 10 min (Eppendorf); the cell pellet was washed by decanting the supernatant followed by suspension in 0.1% peptone water (BD, Thermo Fisher Scientific). The culture was washed twice in this manner and finally resuspended in 5 mL 0.1% peptone water. After preparation of the inoculum cocktail, serial dilution and plating onto appropriate selective media (CCDA (BD, Thermo Fisher Scientific) *C. jejuni*), EMB for *E. coli* was used to determine the starting concentration of the inocula. Three individual colonies were used to create separate inoculum allowing for three biological replicates to be compared during the shelf-life trial.

Inoculation of Kale

Kale was collected from a local producer (Charlotte, Virginia), and the experiment commenced within 24 h of harvest. Kale was stored in plastic bags and placed on ice during transport. The kale was sorted to remove any broken or damaged leaves, and then weighed out into 25-g allotments. For the inoculation of the kale, the 25-g allotments of kale leaves were placed in a weigh boat and a spot inoculation protocol was used to deposit 100 ul of the *Campylobacter* or *E. coli* O157:H7 culture across the kale leaf surface in 10 ul drops. Kale leaves were not coinoculated with both *C. jejuni* and *E. coli* O157:H7. The inoculated kale leaves were placed within a biosafety cabinet for ca. 1 h until the droplets dried. All kale samples were then placed into sterile whirl pack bags (Nasco). A total of 15 samples were inoculated with *Campylobacter* per replicate (45 total), 15 were inoculated with the *E. coli* cocktail per replicate, and 15 were left uninoculated per replicate. All bags were held at 4°C, ±2°C once stabilized, in plastic-lidded containers that also contained an open bottle of water to control humidity to an average of 95% humidity (ranged from 86.5% to 100%). A temperature and humidity tracker

(Vertiv) was also placed with the uninoculated kale samples. The inoculated kale leaves were destructively sampled at day 0 (after drying), 1, 2, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and 23 (14 time points).

Microbiological Sampling and Enumeration

For the sampling process, one bag of kale inoculated with the *E. coli* and *C. jejuni* from each of the replicates (n = 3 for each microbe per time point) and one bag of noninoculated kale were removed from 4°C storage and 225 mL of 0.1% peptone water was added to each bag. The bags were placed in a lab blender (Bagmixer 400, Interscience) for 1 min at 230 RPM. After one minute, the bag was hand massaged for 30 s and blended for an additional minute. Samples were serially diluted tenfold in 0.1% sterile peptone and plated onto Tryptic Soy Agar (BD DIFCO, Franklin Lakes, NJ) and the appropriate selective media (CCDA for *C. jejuni* and EMB Agar for *E. coli*). The noninoculated sample was serially diluted in 0.1% sterile peptone and plated on all three media types. An additional 1 ml of the 10−1 dilution was taken and enriched in 9 mL Bolton Broth without blood or Brilliant Green Bile Broth 2% (Himedia) for *C. jejuni* and *E. coli*, respectively. The enrichments were held at the same temperature as the plates and after 24 h, a loopful was streaked onto either CCDA incubated at 37°C in 10% CO_2 for 48 h or EMB at 37°C for 24 h. On the CCDA, only small, flat, and gray colonies were counted as *C. jejuni*. On the EMB, only colonies with a green sheen were counted as *E. coli*. All colony types were counted on the TSA. The non-inoculated control plates did not contain colonies with characteristic *E. coli* or *C. jejuni* appearance when plated on EMB or CCDA, respectively. The limit of detection for serial dilution and plating is 1 log CFU/g.

PCR Screening of Enrichments

Additionally, at day 5 and each time point, 1.5 ml of the enrichment from the *C. jejuni* and *E. coli* samples were extracted with the Biobasic EZ-10 spin column soil DNA Minipreps kit (Biobasic) and screened with the corresponding PCR protocol to further enhance detection when cell numbers were below the 1 log CFU/g limit of detection by serial dilution and plating. The limit of detection for enrichment and PCR is ~0.27–0.67 log CFU/g.

Primers C412F and C1228 R were used to detect the inoculated *Campylobacter* from the enrichments (Linton et al., 1996). DNA (2 µL) extracted from the Bolton broth enrichments were amplified in 25 µL reactions containing 12.5 µL Gotaq Green Master Mix 2X (Promega), 1 µL magnesium chloride 25 µM (Qiagen), 7.5 µL nuclease-free water (Qiagen), and 0.2 µM of each primer (Integrated DNA Technologies). Nuclease-free water was used for the negative control. DNA from *C. jejuni* ATCC 33291 was used for a positive control. PCR conditions were 95°C for 2 min. followed by 35 cycles of 95°C for 2 min, 57°C for 1 min, 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 1× Tris-acetate-EDTA buffer stained with ethidium bromide. The gel was visualized under a UV transilluminator (Bio Rad).

A multiplex PCR was used for the detection of *E. coli* using the phoA gene designed to detect members of the *E. coli* species (Kong et al., 1999) and eae designed to detect Enterohemorrhagic pathotypes (Vidal et al., 2005). 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 2X, 1 µL magnesium chloride 25 µM, 7.5 µL nuclease-free Water, 2 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 for positive controls. The mPCR conditions were 95°C for 5 min. followed by 28 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were electrophoresed on 2% agarose gel using 1× Tris-acetate-EDTA buffer stained with ethidium bromide. The gel was visualized under a UV transilluminator. Bands of corresponding size were excised and prepared for sequencing using a gel extraction kit before DNA sequencing.

Experimental Design and Statistical Analysis

The experiment was conducted in triplicate using three independent biological replicates. 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, and "ggplot2" to construct the graph. A linear regression was used to look at the relationship between the day (time point) and log CFU. Relationships were considered significant at $P \leq 0.05$.

Results

The initial levels of *E. coli* on the inoculated kale were 4.8 log CFU/g and after 13 days could no longer be detected by serial dilution and plating on EMB (Fig. 1a). No colonies with characteristic metallic green sheen on EMB were observed on the noninoculated control leaves throughout the experiment (23 d). *E. coli* O157:H7 were intermittently detected by enrichment and PCR until day 19 but overall were not detected at the end of the study. *C. jejuni* recovered from day 0 inoculated kale was 1.8 Log CFU and was detected by serial dilution and plating on CCDA through day 7 (Fig. 1b). *Campylobacter* was undetectable through PCR and enrichment

from day 13 onward. *E. coli* or *Campylobacter* were not detected by serial dilution or plating methods or by PCR on noninoculated kale.

Aerobic bacteria (heterotrophic plate counts, HPCs) associated with the freshly harvested kale increased gradually during storage (Fig. 1c). No significant relationship was observed between HPC log CFU and day ($P < 0.9$). At d 17, some wilting and color changes were observed at the edges of the leaves. By d 23, all leaves were visually judged by researchers MP and AB, no longer acceptable due to their yellow color and wilted appearance. Despite the appearance change and increase in HPC, there was no mold, exudate, or blackening observed.

Discussion

Campylobacter and *E. coli* counts both declined in refrigerated storage over the duration of the shelf-life study. At the end of 23 days of storage at 4°C, neither *C. jejuni n*or *E. coli* were detected through plating, enrichment, or PCR. Growth of the pathogens would not be expected since neither is psychotropic; therefore, storage conditions would prevent the growth of *E. coli* and *Campylobacter sp*. Aerobic heterotrophic plate counts started at 4.8 ± 0.53 log CFU/g and increased to 6 Log CFU which is consistent with an increase in psychrotrophic bacteria growing at 4°C (Xylia et al., 2021). In this study, the initial aerobic mesophilic bacteria load is lower compared to other reported studies (Mansur & Oh, 2015). This may be due to difference in cultivar, temperature, and environmental conditions. Previous studies have also purchased kale from supermarkets where the time from harvest was not provided. In this study, kale was obtained from the field and used within 24 h of harvest. A lower starting inoculum and fresher kale may explain why little evidence of bacterial spoilage was seen until d 19, despite other studies reporting shorter shelf lives of kale (Mansur & Oh, 2015).

Die-off behavior of the two pathogens on kale during refrigerated storage varied. *E. coli* counts were higher over the course of the study than the *C. jejuni* counts. The inoculum for both pathogens applied to the kale was 4.0–4.5 Log CFU; after drying, the recovery of *C. jejuni* was reduced by 2.2 ± 0.76 log CFU but no significant change in *E. coli* occurred after inoculation. The decrease in *C. jejuni* was likely due to exposure to atmospheric levels of oxygen during drying before packaging. Increased oxygen concentrations have been reported to reduce *Campylobacter* survival (Thomas et al., 2020). This method of inoculation has been used in other studies examining the survival of *E. coli* O157:H7 on leafy greens and has not been reported to significantly decrease *E. coli* O157:H7 immediately after drying ((Lopez-Velasco et al., 2010) Mansur and Oh, 2015, Kang and Song, 2017). *E. coli O157:H7* log CFU on unpacked Romaine lettuce has been reported to decrease by only 0.6 log over 8 days (Bhullar et al., 2021). In comparison to this study, the *E. coli* reduced 0.9 logs over 7 days persisting until day 19. Interestingly, one-third of the samples here on day 19 tested positive for *E. coli* by PCR but 0/3 samples were positive on day 17 indicating that persistence is variable. Increased wilting and yellowing were visible on day 19 kale leaves, potentially providing additional nutrients for stress response and repair. Previous studies in minimally processed romaine lettuce have demonstrated that lettuce deterioration was a significant determinant in STEC *E. coli* O157 survival in cold storage (Leonard et al., 2021). It may also be possible the *E. coli* O157:H7 cells themselves are dead but the DNA has persisted on the kale.

Despite *Campylobacter's* shorter survival period during storage, it should still be considered a hazard when it comes to fresh refrigerated products, like kale. Low temperatures may aid the survival of *Campylobacter.* For example, survival of *C. jejuni* in water at 4°C improved to 29–60 days compared to 4–7 days at 25°C (González & Hänninen, 2012). *C.*

jejuni reductions on refrigerated minced beef and chicken stored in plastic bags were 1 log reduction in 6 days; in contrast when stored at 22°C, only 1 day was needed for the same 1 log decrease (Barrell, 1984). In addition to prolonging survival, temperature also plays a factor in cells entering the viable but nonculturable state (Wei & Zhao, 2018).

Enrichment and PCR confirmations were run when target colonies could no longer be detected on the appropriate media. Even though the limit of detection in this study was low at 1 log CFU/g, viable but nonculturable bacteria needed to be considered. Many bacteria, including *C. jejuni* and *E. coli* O157:H7, enter a VBNC state when put under stressful conditions, like the refrigerated temperatures in this study (Zhao et al., 2017). Although undetectable through traditional culturing methods, pathogenic VBNC bacteria still have the ability to result in illness. Enrichment and PCR likely allowed us to detect fewer than 10 CFU of stressed but viable bacteria. It is possible that if the bacteria were VBNC, the limit of detection was higher because growth did not occur during the enrichment step; therefore, requiring more samples. The presence of VBNC bacteria may explain the variability among the replicates in the later days of the experiment since the small number of surviving but not growing bacteria would be difficult to detect with traditional PCR.

Environmental point source contamination and produce handling may result in different starting contamination or survival dynamics than observed in this study. Previous studies have demonstrated a 1.23 log CFU reduction in *E. coli* O157:H7 when kale was washed with water at 55°C and subsequently refrigerated (Kang & Song, 2017). This compares to the 0.6 log CFU reduction in this study, with no washing, from day 1 to day 2, suggesting that washing may be a useful additional step to reduce initial contamination. Quantification of *E. coli* from naturally contaminated leafy greens has reported populations around 1 log CFU (Johnston et al., 2005);

however, naturally occurring populations of *Campylobacter* on produce have not been reported to the best of our knowledge. It is likely that the initial inoculum chosen for this study to facilitate quantification exceeds that which would be expected on kale due to field contamination and would therefore represent a worst-case scenario.

The microbial load of *C. jejuni* and *E. coli* on fresh kale reduced over time in a temperature and humidity-controlled environment but was still present when the product would still likely be consumed. Due to this, it is still important to practice safe sanitation management when handling raw leafy greens as higher pathogenic counts have been observed, especially in summer months (Xylia et al., 2021). In this study, the kale was not washed in chlorine prior to inoculation and was refrigerated under high humidity as recommended for extended shelf-life. These results cannot be applied to situations where refrigeration is not used for storage, which may include informal markets or other locations where refrigeration is not available. *E. coli* O157:H7 has been documented to grow on kale at temperatures above 25°C (Mansur & Oh, 2016). This highlights the need for good agricultural practices and proper postharvest handling. Future work looking at the growth of *Campylobacter* spp. on vegetables at different storage conditions and different packaging configurations is needed.

Author Attribution

Auja Bywater: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kathleen Alexander:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Joseph Eifert:** Writing – review & editing, Conceptualization. **Laura K. Strawn:** Writing – review & editing, Software, Resources, Data curation, Conceptualization. **Monica A. Ponder:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

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Conflict of Interests

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.

Research Table

Table 1: A list of the *Escherichia coli* primers used and their associated properties

Gene	**Sequence (5' to 3')**	**Size (bp)**	**Target bacteria**	**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	TACACTGTCATTACGTTGCGGATTTGGC GT		*E. coli control*	0.24 μM	

Research Figure

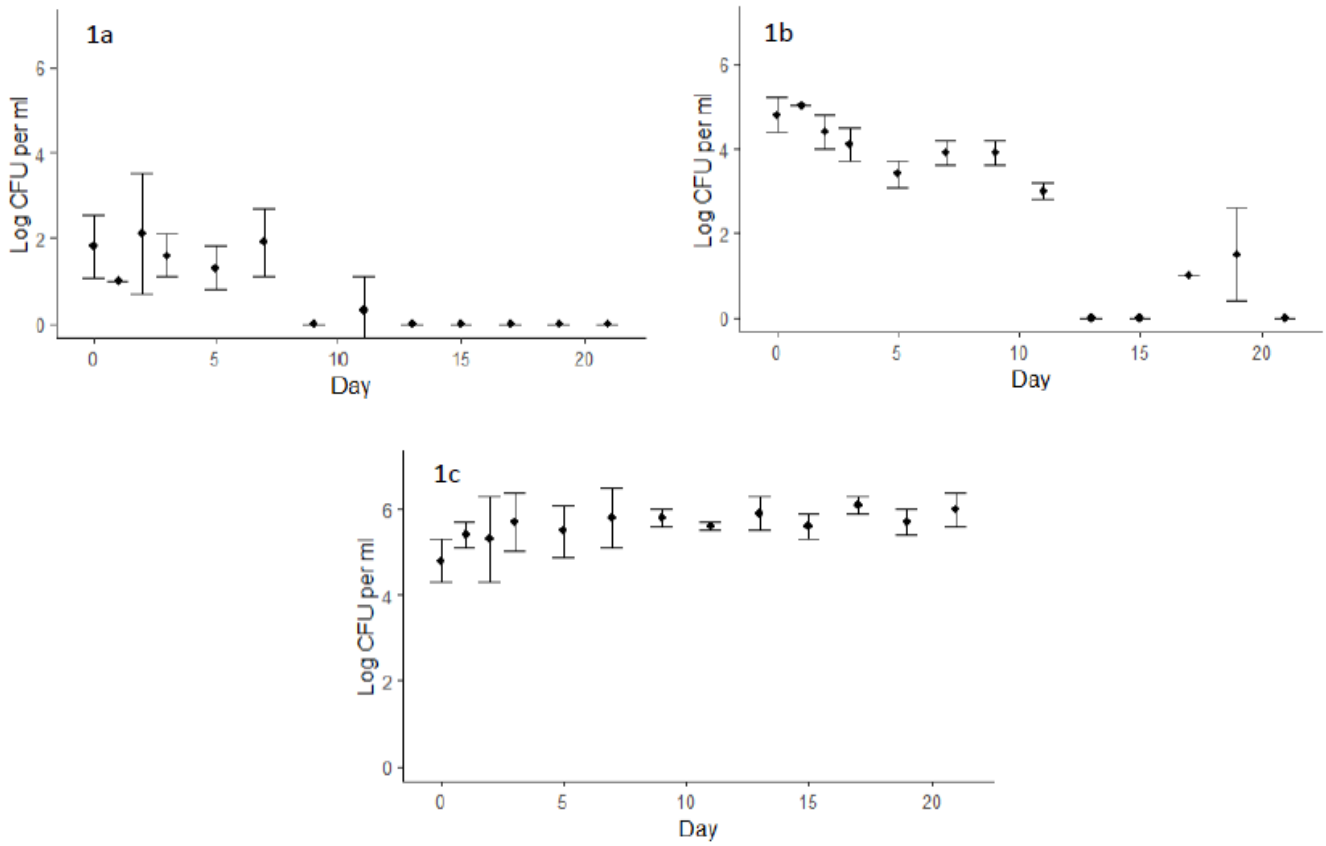

Fig. 1. Effect of time on Log CFU/g of *C. jejuni* (1a), *E. coli O157:H7 (*1b), and aerobic heterotrophic bacteria (1c) from kale stored at 4C. Values shown are the average of all three replicates. Error bars represent the standard deviation among the three replicates. HPC are from TSA, *Campylobacter* counts are from CCDA, E. coli counts are from EMB.

Supplemental Materials

Supplemental Table 1: The daily average for Temperature and Relative Humidity over the 22-day course of the experiment.

Day	Temperature (C)	SD (Temp)	Relative Humidity (%)	SD (RH)
1	11.7	13.3	87.8	4.3
2	5.1	0	92.6	4.1
3	5.1	0.3	94.4	2.8
4	4.9	0.4	95.4	2.8
5	5.1	0	94.6	3.5
6	5.1	0.2	94.6	2.8
7	5	0.4	96.2	1.6
8	5.1	0.3	95.0	2.6
9	5.1	0	96.1	1.8
10	5.1	0	95.5	2.3
11	5.1	0	95.9	2.8
12	5.1	0.3	94.4	2.7
13	4.9	0.4	96.5	1.5
14	5.1	0.3	94.8	2.5
15	4.8	0.4	96.2	1.7
16	5.1	0.3	92.8	3.7
17	5.1	0	94.1	3.6
18	5.4	4.6	94.6	2.8
19	4.7	0.3	97.1	1.9
20	4.8	0.4	95.6	3.1
21	5.1	0	94.7	2.5
22	5.1	0	96.3	1.8

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Chapter 4:
Characterization of *E. coli* and *Salmonella* in Food, Food Waste, and Water from Botswana

Methods

Food, Food Waste, and Water Sample Collection and Processing in Botswana

Beef, pork, poultry, leafy greens, intact fruits, and vegetables were obtained from three different markets (formal and informal) in Kasane, Botswana. Samples were collected monthly during 2022. All produce and meat samples screened were free from visible mold or decay. All samples were processed within 24 hours of collection. After processing, produce samples were frozen and shipped to Virginia Tech for further processing. Meat and poultry samples were screened, and only purified isolates were shipped to Virginia Tech to comply with U.S. Department of Agriculture Animal and Plant Health Inspection Services, Veterinary Services requirements (Permit 610-22-272-03465) to prevent importation of viruses that would threaten US livestock.

A. Produce and Meat 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 25g pieces, and placed in a sterile filter bag. The processed produce samples then had 50mL of 0.1% sterile peptone (Becton, Dickinson and Company, Sparks, Maryland) added into the filter bags. The meat samples were processed according to the

following. Chicken feet and chicken pieces were weighed into 50g allotments in filter bags with 50 mL of 0.1% peptone buffer added. Mince and bone meal were weighed into 50g allotments and placed in a filter bag with 100mL of 0.1% peptone added. The chicken intestines (mala) had 30mL of sterile peptone pushed through them with a sterile syringe: the liquid was dispensed into a filter bag with the remaining intestine pieces. Once processed as outlined above, all meat and produce samples were placed on the orbital shaker (Oxford Lab Products, San Diego, California) for 10 minutes at 2000 rpm.

The peptone rinse from the processed samples was decanted into two 15mL falcon tubes and centrifuged (Axiology Labs, Gauteng, South Africa) at 3000 rpm for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 13mLs of the appropriate selective media: Brilliant Green Bile Broth (BGBB) (Becton, Dickinson and Company) for *E. coli*, Bolton Broth (BB) (ThermoFisher Scientific, Waltham, Massachusetts) for *Campylobacter*, and Rappaport Vassiliadis Broth (RV) (Becton) for *Salmonella.* The BGBB and RV were incubated at 42°C for 4h and then at 37°C for 20h. The Bolton Broth was placed in a MiDI 40 series incubator (Thermo- Fisher Scientific) at 42°C for 4h and then at 37°C for 20h, in 10% CO_2. After incubation, the produce enrichments were centrifuged for 10 minutes at 2000 rpm to pellet the cells. The pellet was resuspended in 250 µL of sterile 0.1% peptone and dispensed into two 1.5mL culture tubes with Tryptic Soy Broth (Becton, Dickinson and Company) with 20% glycerol. All meat isolates were processed in Botswana according to the “Bacterial isolation” section below immediately after enrichment. The produce pellets were frozen and sent to Virginia Tech for bacterial isolation.

B. Environmental Water Collection and Processing

Every two weeks water samples were collected from predetermined transect locations of the Chobe River. Collection points were 1km apart, starting 200m from the ferry at Kazungula and ending in the Chobe National Park. Water was collected from these points approximately 10m from the riverbank using sterile glass containers and stored at 4°C until processing. Samples were processed within 24 hours of collection using a modified USEPA method 1103.1 and method 1604. Volumes were determined based on previous results at each transect and filtered through a 0.45 um bacterial filter. The filter was placed on RAPID *E.coli*2 agar plates (BioRAD, Hercules, CA) and incubated at 37°C for 2h and 44°C for 16-22h. All water isolated *E. coli* were then processed in Botswana according to the "Bacterial isolation" section below.

C. Bacterial Isolation and DNA Isolation

Further bacterial isolation was done for *E. coli* and *Salmonella* from meat, produce, and water. For *E. coli* from meat and produce, BGBB enrichments were plated onto Eosin Methylene Blue (EMB) (Becton, Dickinson and Company, Sparks, Maryland) and incubated at 37°C for 24h. Colonies with a green sheen, indicative of *E. coli*, were passaged at least four times for isolation. For *E. coli* isolated from water, purple colonies, indicative of *E. coli*, were streaked for isolation and purity via 4 passages on EMB followed by Macconkey Agar (Becton, Dickinson and Company). Colonies with a green sheen were selected from EMB and bright pink colonies were selected from Macconkey. All *E. coli* isolates from water were passaged at least four times for isolation. For *Salmonella*, RV broth enrichments were plated onto xylose lysine deoxycholate agar (Becton, Dickinson and Company) and incubated at 37°C for 24h. Black colonies on XLD agar were passaged at least four times before PCR confirmation of *Salmonella* using *invA*

primers.(Malorny et al., 2003). A minimum of four isolates for each microbe type were chosen and preserved in tryptic soy broth with 20% glycerol.

D**. Produce Pellet Enrichment and DNA Isolation**

The enriched vegetable pellets received from Botswana were received and frozen at 0˚C until processing. For processing, the sample was thawed and vortexed to resuspend the cells. A 50 µL aliquot was removed and added to 9mL of Brilliant Green Bile Broth 2% (Himedia, Nashik, India) and BB with blood (ThermoFisher Scientific), and RV Broth (Becton). The Brilliant Green and RV enrichments were incubated at 37°C for 24h. The Bolton Broth enrichment was incubated at 42°C for 4h and then at 37°C for 44h in a MIDI 40 series CO_2 incubator (Thermo Fisher Scientific) at 10% CO_2. Characteristic green sheen colonies (EMB), black colonies (XLD), grey colonies (CCDA) were selected and restreaked for isolation a minimum of four times. Four isolated colonies were grown up in the appropriate media: Brilliant Green for *E. coli*, Rappaport Vassiliadis for *Salmonella*, and Bolton Broth without blood for *Campylobacter*. They were centrifuged at 10,000 rmp for 5 minutes, the supernatant was discarded, and the isolates were resuspended in 0.1% peptone water. This was repeated twice to wash the pellet. The pellet was resuspended a final time in Tryptic Soy Broth (Becton, Dickinson and Company) with 20% glycerol and distributed into 1.5 mL aliquots into cryovials for storage at -80°C. DNA isolation was completed for all *E. coli* isolates by following the manufacturer's instructions for the Biobasic EZ-10 Spin Column Soil DNA Mini-Preps Kit (Biobasic, Amherst, NY). DNA extraction was conducted from 1mL of the Bolton Broth enrichments by following the manufacturer's instructions for the Zymo Fecal DNA MiniPrep Kit (Zymo, Irvine, CA). DNA isolation was completed for *Salmonella* isolates by heating an isolated colony suspended in 100 µL of nuclease-free water for 30s in the microwave.

PCR Detection of *Campylobacter* Genes

The produce pellet Bolton Broth enrichments were screened for *Campylobacter* through mPCR. This was done to catch any VBNC *Campylobacter* that may not have been culturable due to the shipping times and conditions. The Bolton Broth enrichments were only performed on 65 produce samples collected from January-March of 2022. The mPCR protocol contained a primer to detect all members of all *Campylobacter* species in the genera and was composed of three specific pairs of primers. One pair for the amplification of the C412 and C1228 region of 16S rRNA gene designed to detect members of all *Campylobacter* species in the genera. The other two pairs target species-specific regions of *hipO* and *aspartokinase* designed to detect *C. jejuni* and *C. coli* respectively. The sizes of the expected mPCR products and concentrations of the primer sets and pairs are listed in Table 1.

The mPCR amplification was conducted in a total reaction volume of 25µL containing 12.5µL Gotaq Green Master Mix 2X (Promega, Madison, Wisconsin), 1µL Magnesium Chloride 25µM (Qiagen, Germantown, Maryland), 7.5µL Nuclease Free Water (Qiagen, Germantown Maryland), 2µL of DNA template, and the corresponding primers (Integrated DNA Technologies, San Diego, California). Nuclease-free water was used for the negative control. *Campylobacter coli* ATCC 35223 and *Campylobacter jejuni* ATCC 33291 (ATCC, Manassas, VA) were used for positive controls.

PCR Detection of *E. coli* Genes

A multiplex PCR was used for the detection of *E. coli* using the *phoA* gene designed to detect members of the *Escherichia coli* species (Kong et al., 1999), *eae* designed to detect Enterohemorrhagic and Enteropathogenic pathotypes, and *est*1b to detect Enterotoxigenic *E. coli*

(Vidal et al., 2005). The sizes of the expected mPCR products and concentrations of the primer pairs are listed in Table 2. The mPCR amplification was conducted in a total reaction volume of 25 µL containing 12.5 µL Gotaq Green Master Mix 2X, 1µL Magnesium chloride 25µM, 7.5 µL Nuclease Free Water, 2 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 (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 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 2. 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 2) 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. The expected size of the *invA* gene was 123 bp. PCR 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, 2 µL of cellular extract, and at 0.4µM *inva* primers (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 one minute, 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.

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 20/10µg (AMC), ampicillin 10µg (AM), azithromycin 15µg (AZI), chloramphenicol 30µg (CHL), cefotaxime (CTX), ciprofloxacin 5µg (CIP), doxycycline30µg (DOX), gentamicin 10µg (GEN), meropenem 10µg (MER), streptomycin 10µg (STR), tetracycline 30µg (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 break points 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 resistant to five or more classes of antibiotics. Two isolates were screened from each *phoA+* food sample.

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, and "ggplot2" to construct the graphs. The data was not normally distributed requiring non-parametric tests. A Fisher's Exact test was used to determine if there was a significant association between *E.* coli presence and source attributes including: vendor/source, season, quarter, food group, and food handler status. An independent T-test was used to determine if there was a significant association between *E. coli* genes and rainfall data. A two-way T-test for proportions was used to compare the numbers of multiclass-resistant isolates of produce against beef, poultry, and pork. Rainfall data was collected for the Kasane region of Botswana from the Environment and Tourism Ministry in the Republic of Botswana. Relationships were considered significantly dependent at $P \leq 0.05$.

In order to group the wide variety of food samples received, they were broken down into broader sample types for analysis. The Fruit Group contains pear, pineapple, orange, apple, banana, pawpaw, grapefruit, watermelon, and lemon. The Greens group contains spinach, collard greens, rape, cabbage, broccoli, and lettuce. The Above Ground group includes tomato, corn, maize, and yellow, green, and red pepper. The Below Ground group includes carrots, potatoes, and onions. The Soil Associated group contains cucumber, celery, butternut squash, and pumpkin. The meat samples were grouped by the animal they came from.

Results

1. Produce

A total of 294 produce samples were screened. *E. coli* was isolated from 20% (60/294) of samples and *Salmonella* from 2% (7/294) of samples. *Campylobacter* was not detected in any of the Bolton Broth enrichments (0/65). From these positive samples, 183 *E. coli* and 28 *Salmonella* isolates were collected. *E. coli* isolates were confirmed by the presence of *phoA* and *Salmonella* by the presence of *invA* genes. Among the *E. coli* isolates, 2% (3/183) had the *eae* gene but were negative for *stx1* and *stx2* genes. The *eae* positive samples came from apples, carrots, and cucumbers. All three of these samples came from vendor A collected in August 2022, although the distributor vendor A obtained them from is unknown. None of the isolates were identified as having the *est1b* gene.

Greens (27.4%) and soil-associated vegetables (23%) were the two produce groups with the most *E. coli* isolated (Figure 1). *Salmonella* was only isolated from 3 fruit samples, 1 above ground, 2 below ground, and 1 soil associated sample (Figure 1). The presence of *E. coli* (P = 0.53) and *Salmonella* (P = 0.15) was not influenced by produce sample type.

Samples were collected from two local grocers, Vendors A (N = 84) and B (N = 104), a local Vendor C (N = 4), and the landfill (N = 102). *E. coli* was isolated more often from the landfill than the vendors (P = 0.01) while *Salmonella* isolation (P = 1) was not influenced by source (Figure 2). *Salmonella* was isolated only from produce from Vendor B (4.7%) and Vendor A (2.3%).

E. coli isolation from produce was not impacted by time of the year during which samples were collected (quarter, P = 0.6) (Figure 3). *Salmonella* isolation from produce occurred only in

samples collected from October to December (P < 0.001). *E. coli* isolation (P = 0.15) and *Salmonella* isolation (P = 0.7) were not impacted by time of year when samples were collected (season) (Figure 4). No significant association was found between rainfall during time of collection and *E. coli* isolation from produce (P = 0.9).

2. Beef, Poultry, and Pork

A total of 138 beef, poultry, and pork samples were screened. *E. coli* was isolated from 49% (68/138) of samples. From these positive samples, 301 isolates were collected. *Salmonella* and *Campylobacter* were not isolated. Only one isolate obtained from chicken intestines was positive for *eae,* but negative for *stx1* and *stx2.* None of the isolates were identified as having the *est1b* gene. *E. coli* Isolation frequency exceeded 40% for beef and chicken (Figure 1). The meat type was not associated with *E. coli* isolation (P = 1). *E. coli* was isolated most frequently from meat samples from Vendor B (Figure 2). *E. coli* was isolated more often from samples obtained from vendors than the landfill (P=0.04).

E. coli was isolated from 52.3% of samples from April to June and 54.3% of samples from July to September (Figure 3). From the beef, poultry, and pork samples collected during the dry season, 54% of them had *E. coli* isolation (Figure 4). There was no significant association between *E. coli* isolation and the time period the samples were purchased (quarter of year, P = 0.7) or the season when samples were purchased (wet and dry season, P = 0.16). *E. coli* isolation from beef, poultry, and pork was not associated with time period when the sample was collected (quarter of year) (Table 7, P = 0.7). There was no significant difference in the number of *E. coli* isolated from beef, chicken, or pork in the wet season compared to the dry season (Table 8, P = 0.16). There was also no significant association between *E. coli* isolation from beef, poultry, and pork and rainfall during the time period of collection (P=0.2).

3. Water

A total of 432 water samples were collected. Isolation and screening for *S. enterica* and *Campylobacter* were not performed for water samples. *E. coli* was isolated from 84.7% (366/432) samples; from these positive samples, a total of 2304 isolates were collected. Out of the isolates, 2% (47/2304) had the *eae* gene, all but one of them also had the *stx1* gene while none of them contained *stx2*. Furthermore, 0.2% (5/2304) contained the *est1b* gene.

There were 108 water samples collected from each quarter. *E. coli* isolation (P = 0.001) and *eae* presence (P < 0.001) was associated with time period (Quarter, Figure 3). There was no significant association between the *est1b* gene presence and the time period the water was collected (quarter, P=1).

There were 216 samples collected during the wet and dry seasons as outlined in Figure 4. The *eae* gene was only found in *E. coli* isolates collected during the wet season and its prevalence was significantly correlated with season (P<0.001). *E. coli* isolation (P=0.14) and *est1b* presence (P = 1) was not significantly impacted by time when the water was collected (wet and dry season). There was a significant association between *E. coli* isolation (P=0.02), and *eae* prevalence (P=0.02) and rainfall amount at the time of collection, with higher gene prevalence being found in months with more rain. There was no significant association between *est1b* prevalence and rainfall amount at the time of collection (P=0.6).

Of the water samples, 19.4% (84/432) were collected at times when recreational boats passed through the water. From those samples, *E. coli* was isolated from 81% (16/84) and of those isolates, 5.6% (5/84) contained *eae*, and 1.2% (1/84) contained *est1b*. Recreational boats passing

through the water areas didn't have a significant impact on isolation of *E. coli* (P=0.4), presence of *eae* (P=0.57), or *est1b* genes (P=0.35).

4. Susceptibility of Food and Food Waste Associated *E. coli* and *S. enterica* Isolates to Twelve Different Antibiotics

Antibiotic-resistant *E. coli* were isolated more commonly from beef, poultry, and pork than in produce (Figure 5). The *E. coli* isolates from produce were most often resistant to ampicillin and tetracycline. Antibiotic resistance was most found to ampicillin, doxycycline, trimethoprim/sulfamethoxazole, and tetracycline for *E. coli* isolated from beef, poultry, and pork. No isolates were resistant to meropenem. *E. coli* isolates from poultry were most often resistant to tetracycline followed by doxycycline. *E. coli* isolates from the Below Ground and Pork groups were only resistant to one antibiotic (Table 3). *E. coli* isolated from produce from July to September or during the dry season were often resistant to amoxicillin (Tables 4, 5). *E. coli* isolated from beef, poultry, and pork during January-March and the wet season were often resistant to Trimethoprim/Sulfamethoxazole (Tables 4, 5).

Out of the produce *E. coli* isolates screened, 15.5% (16/103) were multidrug and multiclass resistant. Out of the beef, poultry, and pork *E. coli* isolates screened, 23.6% (37/157) were multidrug-resistant and 22.2% (35/157) were multiclass-resistant (Figure 5). The number of multiclass-resistant *E. coli* isolates from produce was significantly different than the number of multiclass-resistant *E. coli* isolates from beef, poultry, and pork (P = 0.04). Multiclass resistance in *E. coli* was associated with type of produce (P = 0.04), but not type of meat (P = 0.6). Multiclass resistance was seen in higher frequency in produce from the vendor and beef, poultry, and pork from the landfill (Figure 6). Multiclass resistance was associated with source for produce (P = 0.03), and beef, poultry, and pork (P<0.001).

E. coli isolated from produce (P <0.01) and beef, poultry, and pork (P <0.01) were more often multiclass resistant for samples collected during months associated with the wet season (Figure 7,8). Isolation of MCR *E. coli* was more frequent from produce (P = 0.01) and beef, poultry, and pork (P<0.001) collected in months with highest rainfall.

All 28 *S. enterica* isolates from produce were tested for antibiotic resistance. Amongst the isolates, only one had resistance to any of the tested antibiotics and this was chloramphenicol. All isolates were susceptible to amoxicillin, azithromycin, ciprofloxacin, cefotaxime, doxycycline, gentamicin, meropenem, streptomycin, trimethoprim/sulfamethoxazole, and tetracycline. There were three isolates with intermediate resistance to ampicillin.

Discussion

E. coli is widely used as an indicator species for fecal contamination in food and water sources (Odonkor & Ampofo, 2013). *E. coli* is also a well-understood organism which allows for established methods and widespread acceptance of findings. Different regulations exist on how many *E. coli* are allowable in food and water based on the type of *E. coli* and whether that product will be subjected to additional lethality treatments before consumption. In the United States, the "Produce Safety Rule" outlines that water used for growing produce must have 126 or less CFU of *E. coli* per 100 mL of water (U.S. Food and Drug Administration, 2022). The Produce Safety Rule also requires that fields must be inspected for fecal contamination during growing seasons to reduce pathogenic contamination. Botswana follows EU legislation that dictates that produce available for sale must be screened for safety. With produce, five samples are to be taken from a produce lot for testing, according to European Economic Community guidelines, for *E. coli* and only two of them can have between 100-1,000 CFU/g (Botswana Investment & Trade Centre). With fresh meat according to the European Economic Community

directives 88 and 657, five samples are to be taken from the lot with no more than two being allowed to contain between 500-5,000 CFU/g (European Economic Community, 1988). There are also guidelines for meat such as 500 CFU/g as a critical limit for raw beef and 50 CFU/g as a critical limit in cooked chicken (U.S. Food and Drug Administration, 2022). While the methods used in this thesis do not quantify the numbers of *E. coli* present in the food, they do provide perspective on the contributions of food and water to the spread of diarrheagenic *E. coli* and *Salmonella* in Botswana.

This study saw the most E. *coli* isolation from green vegetables which, together with other findings, indicates that *E. coli* is commonly found in greens in southern Africa (Richter et al., 2021, 2022). This is likely due to the greens having close contact with soil contaminated via water or animal movements. The Soil Associated category was ranked number two based on the frequency of *E. coli* isolation which could be explained by the same proximity to soil as the Greens group, or the smaller sample size. Finding *E. coli* on produce isn't exclusive to Botswanan grocery stores, it has also been reported in German grocery stores from cilantro, arugula, and mixed salads (Reid et al., 2020). The *eae* gene is present in many diarrheagenic *E. coli*, in particular the enteropathogenic and enterohemorrhagic pathotypes (Hamner et al., 2019). These isolates came from carrot and cucumber samples which would have been in contact with the soil where *E. coli* has been reported to be present in southern Africa (Iwu et al., 2021). This could allow for the transfer of the *E. coli* from the soil to the produce. A low prevalence of *eae*, 1% (2/180), has also been reported in *E. coli* isolates from spinach, lettuce, parsley, carrots, cucumber, and tomato (Özpınar et al., 2013). No *eae* genes were reported in vegetable isolates from Jakarta (n=27), but they were present in *E. coli* isolated from fruit 24% (12/49) with *E. coli* isolates from tomatoes having the only *eae* genes in from tomatoes, starfruit, and guava

(Waturangi et al., 2019). Differences could be attributed to geographical regions or differences in growing conditions including water sources used for irrigation, organic soil amendments, and animal intrusion (Williams et al., 2015). This contrasts with the findings of Iwu et al., where ETEC comprised 67% (4/6) of the confirmed *E. coli* isolates. While limited data is available for *E. coli* from food waste in landfills, the higher incidence rate of *E. coli* in produce from the landfill compared to the other sources isn't surprising as it may be transferred from wildlife, while they scavenge in the landfill for food. While foraging for food it is possible that wildlife excrement containing *E. coli* comes into contact with the food waste. This link between wildlife foraging, and presence of *E. coli* has been reported in other studies looking specifically at migratory birds (Ahlstrom et al., 2018). Further foraging of the contaminated food may further spread the *E. coli* to subsequent food waste in the landfill or to animals consuming the waste.

Compared to this region of Botswana, *E. coli* isolated from food in Jimma town, Ethiopia was reported in 20.8% (35/168) of minced meat and 17.5% (14/80) of swabs collected from the butchering environment in a meat retail shop (Abayneh et al., 2019). The rates in Ethiopia are lower than the findings of this study, potentially due to geographic and cultural differences. A study done in South Korea reported *E. coli* in chicken (79/133, 59.4%), beef (24/133, 18.6%), and pork (10/133, 7.5%) (Cho et al., 2020). This follows the patterns of this study where beef and chicken both contained more *E. coli* than pork. The isolate containing an *eae* gene came from chicken intestines from Source A in August 2022. These findings are supported by another study that found the *eae* gene in 0.6% (13/2,017) of diverse meat samples from all South African provinces and imported meat (Madoroba et al., 2022). These rates could be similar because the chicken sold in Botswana may be imported from South Africa. Another study in Riyadh, Saudi Arabia reported the *eae* gene in 27.27% (3/11) *E. coli* O157:H7 isolates collected from chicken,

raw beef, and mutton (Hessain et al., 2015). These rates are higher than the values observed in this study, this could be due to the smaller sample size. It is interesting to note the lower *E. coli prevalence* in beef, poultry, and poultry from vendors compared to food waste from the landfill, this could be due to difficulty locating scrap meat sources in the landfill.

Each quarter was pretty equal in terms of *E. coli* prevalence in produce and beef, poultry, and pork. Additionally, seasonality saw pretty equal breakdowns of *E. coli* prevalence in produce and beef, poultry, and pork. The lack of significance between *E. coli* prevalence in produce and quarter and season is contrasted by other findings that *E. coli* persisted longer in the soil than compared to lettuce phyllosphere, a variation they say is likely due to weather-related factors, such as temperature and rainfall (Xu et al., 2016). These findings of this study may not be significant due to a large amount of Botswana's fresh produce being grown in other countries and imported. The lack of association between *E. coli* prevalence in beef, poultry, and pork based on season is supported by a study in Colombia that found meat and animal hides had no statistical significance with *E. coli* and the rainy season (Calle et al., 2021).

Isolation of *E. coli* from water samples was largely influenced by factors including season, quarter, and amount of rainfall during the month of sample collection. *E. coli* was most prevalent in water collected during the first quarter of the year, but isolation was not more likely in samples collected from the wet season compared to the dry season. This contrasts with a study in Ghana that reported significantly higher counts of *E. coli* in the wet season ($0.2x10^1$ - 0.7×10^1) compared to the dry season ($0\text{-}0.1 \times 10^1$) (Odonkor & Mahami, 2020). All water *E. coli* isolates containing an *eae* gene were from the wet season. The one isolate without *stx1* was considered enteropathogenic *E. coli.* The first quarter had 20 of the eae+ isolates while the last quarter had 1. The 4.8% prevalence of *eae* genes found among water isolates compares to 16% (14/87) found in

North-West Province, South African water (Chukwu et al., 2019). The water samples in South Africa were collected from rivers but also from water storage containers around clinics. The proximity of the water to clinics could increase exposure to *E. coli* contamination from sick individuals. The two water isolates with the *est1b* gene came from the wet and dry seasons and the first and second quarters. Other ETEC genes, (*It* and *sta)* were reported in 11.4% (19/170) of *E. coli* isolates from environmental waters in Johannesburg, South Africa (Bolukaoto et al., 2021). Increased precipitation has been reported to significantly impact if *E. coli* would be found in water sources in Sub-Saharan Africa (Robert et al., 2021). Seasonality may impact enteric zoonotic diseases by influencing animal and insect populations, agricultural activities, and changing rainfall patterns conditions (Lal et al., 2012). *E. coli* has animal hosts which explains why animal activities and populations can influence disease prevalence. If a produce field is close to an animal hotspot, there is an increased risk of contamination from *E. coli* (Glaize et al., 2021). As a natural result of this relationship, months with increased agriculture output are more likely to see enteric zoonotic pathogens. Increased rainfall can stir up sediments containing *E. coli* in water sources and also contribute to the overland transfer of *E. coli* (Cardoso et al., 2012). It has been shown that, when inoculated onto a plot, as much as 50% of *E. coli* was removed the from overland flow with high water saturation, indicating its persistence in water and soil in overland transfer (Mulrhead et al., 2006). This overland transfer can occur as rainfall impacts the transmission of microorganisms from the environment into ground and surface water systems. Climatic factors play a large, complex part in pathogenic survival.

Salmonella lives in the intestinal tract of animals indicating that *Salmonella* in food may be there as a result unhygienic conditions or contaminated water. Two of the produce samples containing *Salmonella* came from Vendor A while the other five came from Vendor B. The

Salmonella were isolated from pear, carrot, green pepper, cucumber, and apple. Isolation of *Salmonella* from fruits and vegetables is rarely reported in Africa (Al-Rifai et al., 2020). Compared to *Salmonella* prevalence in India of 27.5% (11/140) in spinach, 6.2% (5/80) in cucumbers, and 3.7% (3/80) in carrots, the prevalence found in Botswana is low (Verma et al., 2018). All foods from which *Salmonella* was isolated were collected during quarter three, (July-September). This contrasts with a study reporting that *Salmonella* has been shown to have a strong association to season in Chile with higher detection taking place in spring and summer (Toro et al., 2022). Differences could be due to geographical variations and average weather temperatures. No *Salmonella* isolates were obtained from the beef, poultry, or pork samples. In this study, selective enrichment in Rappaport Vassiliadis was used which is similar to a study that tested 18 raw meat samples in Turkey and also reported no *Salmonella spp.* after the same enrichment step (Öncül & Yıldırım, 2019). This compares to a *Salmonella* prevalence in poultry (2.33%, 7/300) and pork (1.74% 5/287) and a *Campylobacter sp.* prevalence in poultry (17% 17/100) in Italy (Corradini et al., 2021). Corrandini enriched their *Salmonella* samples in non-selective media first which may have aided *Salmonella* recovery explaining the higher prevalence.

C*ampylobacter* was not isolated in this study, despite its common association with meat and poultry. *Campylobacter* screening was conducted for the full 12 months only for meat in Botswana. For produce samples, 65 produce Bolton Broth enrichments were screened for *Campylobacter* and negative for presence of the screened *Campylobacter* spp. After these samples, no further produce isolates were screened for *Campylobacter* spp. *Campylobacter* is notoriously difficult to grow due to *Campylobacter sp.* being microaerophilic, having slow growth, being easily out competed by other organisms, and forming VBNC states even if they

survive all other challenges (Buss et al., 2020). The lack of positive samples could be due to that challenge and also the potential environmental stresses on the organism from the unpredictable nature of international shipping. It seems unlikely that *C. jejuni* could have survived on produce during shipment since previous studies found it was unculturable after 13 days (Bywater et al., 2023).

Although there are many different antibiotics, they all belong to a handful of classes that affect bacterial growth through different mechanisms. When bacteria are resistant to more than one class of antibiotics, their illnesses become increasingly difficult to treat. Only two new classes have been introduced since 1940 which is alarming when looking at the rise in multiclass-resistant bacteria (Coates et al., 2011). Multi-class resistance in this study was classified as resistance to three or more antibiotic classes. A couple of the antibiotics used belonged to the same class, so looking only by class and not just individual antibiotics helped identify wider and more developed resistance in the isolates tested. The antibiotics in this study were chosen to get a wide variety of different classes while incorporating antibiotics that are commonly administered over the counter in Botswana as well as to food animals. Antibiotic classes that have been reportedly given to cattle, poultry, and pigs in Africa include tetracycline, aminoglycoside, penicillin, macrolides, sulfonamides, and cephalosporin (Kimera et al., 2020). The meat samples in this study had the highest number of *E. coli* isolates resistant to the tetracycline (88 isolates), penicillin (44 isolates), sulfonamide (37 isolates), and aminoglycoside (27 isolates) classes. This aligns with what would be expected due to the antibiotics given to food animals, especially in the Sub-Sahara. Overuse in humans could result in the spread of antibiotic resistance to food and water through excretion of antibiotics and improper sanitation (Bengtsson-Palme et al., 2018). MCR bacteria typically acquire plasmids and other mobile

elements that confer resistance (Grossman, 2016). It isn't surprising that poultry had the most MCR *E. coli* isolates since the antibiotics listed above are routinely administered to food animals in Africa to promote growth and reduce illness. One of the grocery stores, source A, saw a much larger number of MCR isolates for beef, poultry, and pork than the other grocery store, source B. Variations in antimicrobial profiles from different sources isn't unique to Botswana. In Canada, AMR observed varied where British Columbia saw more resistance to quinolones than Ontario and Quebec (Dramé et al., 2020). Beef, poultry, and pork isolates saw higher MCR numbers in the landfill compared to the grocery stores. This could be largely attributed to the small sample size from landfill. Fruit had the second most MCR *E. coli* isolates. Produce isolates saw higher MCR numbers in the local grocery stores and vendor than in the landfill. Resistant *E. coli* can make its way to produce samples through vehicles like irrigation water and fecal-polluted soils (Abakpa et al., 2018). These resistant microorganisms can be consumed by humans to continue the cyclic transmission of resistant bacteria. Tetracycline is commonly available and had a large number of resistant isolates from beef and poultry. Resistance to antibiotic drugs available over the counter has been noted in pathogenic *E. coli* in Southern Africa (Van et al., 2020). There were 171 ARG's reported in food waste in China showing that antibiotic resistance is present in food waste systems (Lin et al., 2022). This is problematic because genes may be passed on to more bacteria through environmental exposure or fecal contamination from individuals who consume these foods.

Antibiotic resistance in *E. coli* from food and water has been shown to be impacted by seasonality (Zdragas et al., 2012). For example, in Germany, seasonal patterns have been associated for chloramphenicol with resistance from wastewater being higher in the summer (27.3%) compared to spring (11.1%), autumn (6.7%), and winter (4.5%) (Schmiege et al., 2021).

However, seasonality has geographic constraints to it. Unlike many areas of the world, Botswana and its surrounding areas aren't broken down into four seasons but into wet and dry seasons. Including rainfall data in future studies could help bridge the difference in seasonal gaps seen around the world. Rainfall data would be helpful to compare the amount of water, a common factor in antibiotic resistance development and spread, in an area regardless of season. Higher levels of antibiotic-resistant genes have been reported in the water from wells in the wet season compared to the dry season (Huang et al., 2019). The changes in antibiotic resistance levels in water by season and rainfall may be transferred to fresh produce or meat-producing animals, further contributing to the spread of antibiotic-resistant microorganisms. It's important to note that the food samples in this study were likely harvested earlier than the month that they were purchased and sampled. The time of harvest, especially in meat samples, could have been from a different season than they were screened. The harvest time data was not available for this study which could confound results presented here due to differences in harvest season from screening time. The produce collected in this study may have also been grown outside of Botswana, so the environmental and rainfall sources could also be different from Botswana due to geographical variations. Different parts of the world will have different seasonal patterns, but that seasonality will influence antimicrobial profiles.

There were several instances where isolates were not resistant to both antibiotics of the same class. Ampicillin and amoxicillin, both beta-lactam class, require different beta-lactamase enzymes to modify the antibiotic, rendering it safe. In this study, 1.9% (2/103) of isolates resistant to ampicillin were also resistant to amoxicillin. *E. coli* have developed mechanisms of resistance against these antibiotics by producing beta-lactamases. These enzymes currently exceed 2800 unique proteins which could explain why some isolates are resistant to ampicillin,

but not amoxicillin despite them having the same target (Galindo-Méndez, 2020). This suggests the presence of plasmids that promote MDR are circulated in the area. Additional monitoring can help increase understanding of these proteins and how they circulate in the environment. Gentamycin and streptomycin are two other antibiotics from the same aminoglycoside class that saw varying resistance from the same isolate. Aminoglycosides work by binding the site where codon-anticodon accuracy is checked in 16S rRNA; resistance mechanisms to these antibiotics also entails enzyme production to modify the drugs efficiency (Davis et al., 2010). Variations in these mechanisms of resistance could explain the isolates being resistant to only one drug compared to another of the same class.

Increasingly, public health officials are concerned due to the discovery of carbapenemase-encoding genes that have been identified from foods in China and Germany starting in 2007 (Köck et al., 2018). Recent studies in the United Kingdom reported *E. coli* isolation from 397 meat and 400 vegetable samples with no carbapenem resistance found (Randall et al., 2017). In this study, no *E. coli* isolates with resistance seen to meropenem were obtained from food or food waste. This is encouraging since meropenem belongs to the carbapenem class, which is used as a last resort when other antibiotics are not effective.

This study identified prevalence of *E. coli* in food and water and *Salmonella* in food from the Chobe region of Botswana. It also identified antibiotic resistance of these bacteria, finding that many, including pathogens, were multiclass resistant. It identified spatial and temporal factors that influence *E. coli* and *Salmonella* presence in food, food waste, and water. It provided a strong background for future research which will bring in human and animal fecal samples. This project will eventually provide a One Health approach to mitigating zoonotic enteric diseases and improving public health in Botswana and its surrounding areas.

Attributions

This research could not have been completed without the dedicated efforts of the Alexander laboratory in Kasane, Botswana. Galaletsang Makgarebe Dintwe prepared the food samples for enrichment and isolated *E. coli* and *Salmonella* from meat samples, Lefang Chobolo screened all meat samples for *Campylobacter*, and Letty Lekone isolated *E. coli* from water samples. Thanks to all the other members of the lab that have assisted with other necessary tasks to upload the data and shipped the samples to Virginia Tech. Kim Waterman provided laboratory assistance. Rachel Saunders, Evan Nuckolls, Madison Fleming and Christine Kelly performed the antibiotic disk diffusion methodology for non-pathogenic *E. coli* isolates. The Fralin Life Sciences Institute at Virginia Tech performed Sanger sequencing on PCR products to confirm the specificity of PCR performed as confirmation in this thesis.

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Research Tables

Table 1: The *Campylobacter* primers used and their corresponding attributes

Primer/Gene	**Sequence (5' to 3')**	**Size (bp)**	**Target Bacteria**	**Concentration**	**Reference**
hipoF	GACTTCGTGCAGATATGGATGCTT	323	*C. jejuni*	0.4 µM	Persson & Olsen (2005)
hipoR	GCTATAACTATCCGAAGAAGCCATCA			0.4 µM	
Aspartokinase - F	GGTATGATTTCTACAAAGCGAG	502	*C. coli*	0.2 µM	Linton (1997)
Aspartokinase - R	ATAAAAGACTATCGTCGCGTG			0.2 µM	
C412F	GGATGACACTTTTCGGAGC	816	*Campy genus*	0.2 µM	Linton (1996)
C1228R	CATTGTAGCACGTGTGTC			0.2 µM	

Table 2: The *E. coli* primers used and their corresponding attributes

Gene	**Sequence (5' to 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	TACACTGTCATTACGTTGCGGATTTG GCGT		*E. coli control*	0.24 µM	
est1b - F	TGTCTTTTTCACCTTTCGCTC	171	*ETEC*	0.2 µM	Chandra, et al. (2013)
est1b - R	CGGTACAAGCAGGATTACAACAC			0.2 µM	
stx1 - F	CGATGTTACCGTTTGTTACTGTCACAG	216	*EHEC*	0.4 µM	Muller et al. (2007)
stx1 - R	AATGCCACGCTTCCCAGAATT			0.4 µM	
stx2 - F	GTTTTGACCATCTTCGTCTGATTATTG AG	343	*EHEC*	0.4 µM	Muller et al. (2007)
stx2 - R	AGCGTAAGGCTTCTGCTGTGAC			0.4 µM	

Table 3: Total isolates resistant to each antibiotic tested by Food Type from isolates collected from Botswana, 2022. Number of isolates screened: Fruit (N=26), Greens (N=26), Above Ground (N=16), Below Ground (N=14), Soil Associated (N=13), Beef (N=74), Poultry (N=74), Pork (N=4).

Food Type	Food Group	AMC	AM	AZM	C	Cip	CTX	D	GM	MEM	S	SXT	TE
Produce	Fruit (N=36)	2	14	1	5	0	5	6	0	0	4	2	13
Produce	Greens (N=26)	0	7	0	0	0	0	5	0	0	4	3	7
Produce	Above Ground (N=16)	0	0	0	0	0	0	1	0	0	0	0	1
Produce	Below Ground (N=14)	0	7	0	0	0	0	0	0	0	0	0	0
Produce	Soil Associated (N=13)	0	6	0	0	0	2	0	0	0	2	0	2
Meat	Beef (N=74)	4	20	1	0	0	0	9	0	0	10	16	18
Meat	Poultry (N=79)	3	16	2	8	4	4	28	4	0	13	21	33
Meat	Pork (N=4)	0	1	0	0	0	0	0	0	0	0	0	0

Table 4: Total isolates resistant to each antibiotic tested by time of year (quarters) from isolates collected from Botswana, 2022. Number of produce isolates screened: January – March (N=62), April – June (N=94), July – September (N=85), October – December (N=54). Number of beef, poultry, and pork isolates screened: January – March (N=42), April – June (N=21), July – September (N=57), October – December (N=18).

Quarter	Sample Type	AMC	AM	AZM	C	CIP	CTX	D	GM	MEM	S	SXT	TE
January - March	Produce	0	9	1	2	0	7	4	0	0	6	4	10
	Beef, Poultry, and Pork	4	22	3	0	3	2	9	2	0	14	24	16
April - June	Produce	2	10	0	0	0	0	1	0	0	0	0	4
	Beef, Poultry, and Pork	2	2	0	2	0	0	14	0	0	0	2	14
July - September	Produce	0	11	0	3	0	0	7	0	0	3	0	8
	Beef, Poultry, and Pork	1	12	0	5	1	2	11	2	0	8	7	16
October - December	Produce	0	3	0	0	0	0	0	0	0	1	1	1
	Beef, Poultry, and Pork	0	1	0	1	0	0	3	0	0	1	4	5

Table 5: Total isolates resistant to each antibiotic tested by time of year (wet and dry seasons) from isolates collected from Botswana, 2022. Wet season: November – March; Dry season: April – October. Number of produce isolates screened: wet season (N=130), dry season (N=165). Number of beef, poultry, and produce isolates screened: wet season (N=51), dry season (N=87).

Season	Sample Type	AMC	AM	AZM	C	CIP	CTX	D	GM	MEM	S	SXT	TE
Wet	Produce	0	12	1	2	0	7	4	0	0	7	5	11
	Beef, Poultry, and Pork	4	23	3	1	3	2	12	2	0	15	27	19
Dry	Produce	2	22	0	3	0	0	8	0	0	3	0	12
	Beef, Poultry, and Pork	3	14	0	7	1	2	25	2	0	8	10	32

Research Figures

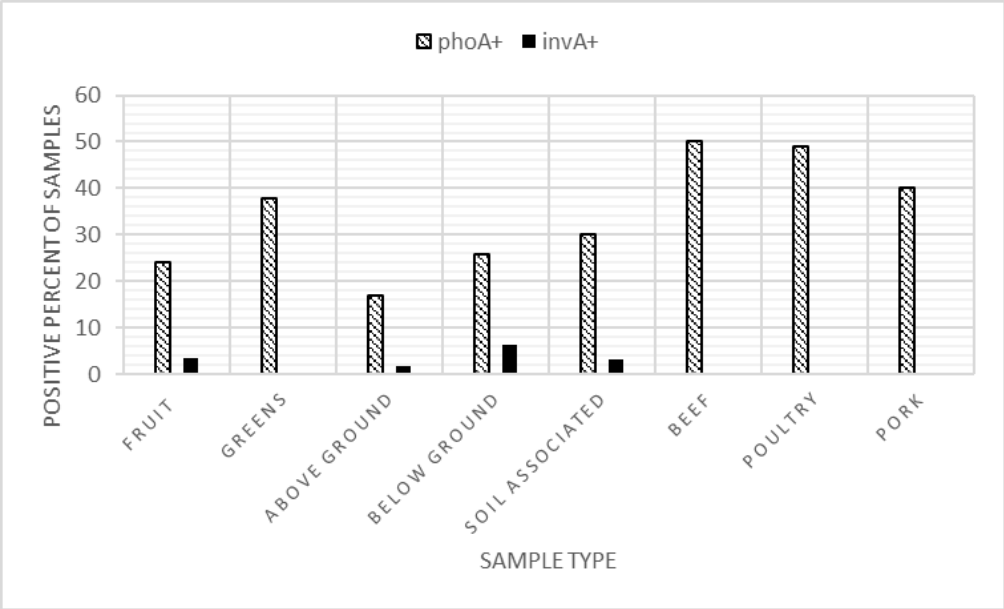

Figure 1: Total samples containing the *phoA* and *invA* gene from different food types collected from the Chobe Region of Botswana, 2022. Fruit (N=103), Greens (N=51), Above Ground (N=62), Below Ground (N=39), Soil Associated (N=39), Beef (N=58), Chicken (N=75), Pork (N=5). No isolates from Greens, Beef, Poultry, or Pork had the *invA* gene.

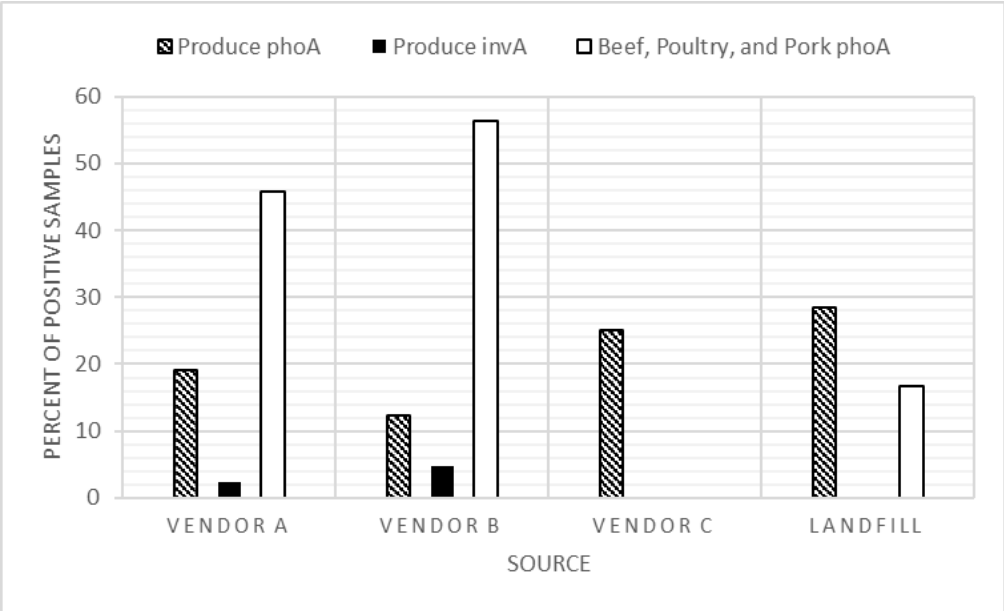

Figure 2: Total samples containing the *phoA* and *invA* gene from different sources in which samples were collected from the Chobe Region of Botswana, 2022. Produce samples: Vendor A (N=84), Vendor B (N =105), Vendor C (N=4), Landfill (N=102). Beef, poultry, and pork samples: Vendor A (N = 48), Vendor B (N= 80), Vendor C (N=0), Landfill (N=6). No *invA* genes were found from Vendor C or the landfill.

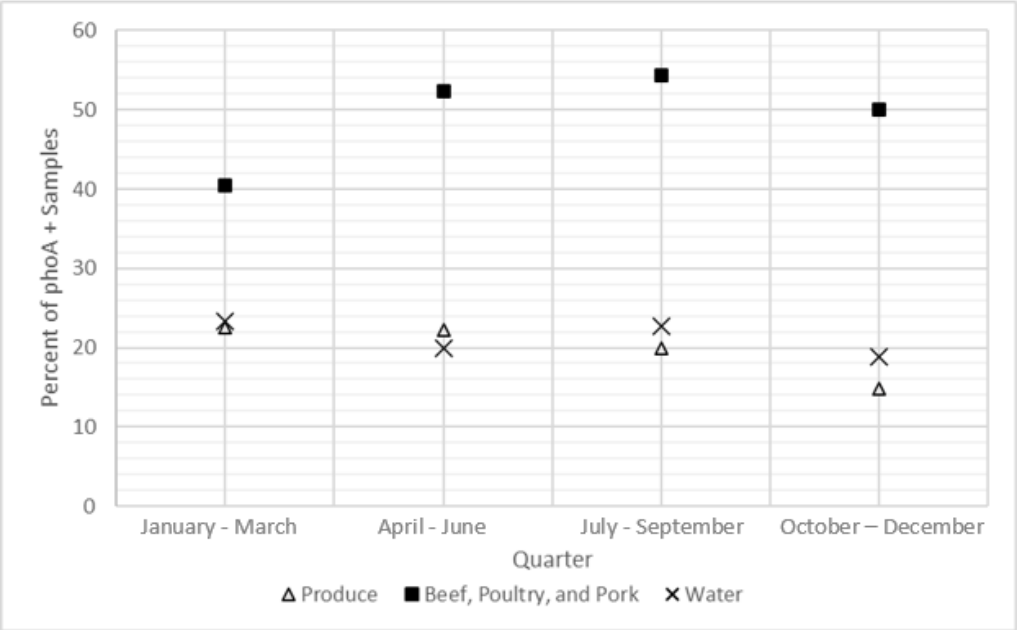

Figure 3: Total samples containing the *phoA* gene from different quarters in which samples were collected from the Chobe Region of Botswana, 2022. Produce samples January-March (N=61), April – June (N=94), July – September (N=85), October – December (N=54). Beef, poultry, and pork samples January-March (N=42), April – June (N=21), July – September (N=57), October – December (N=18).

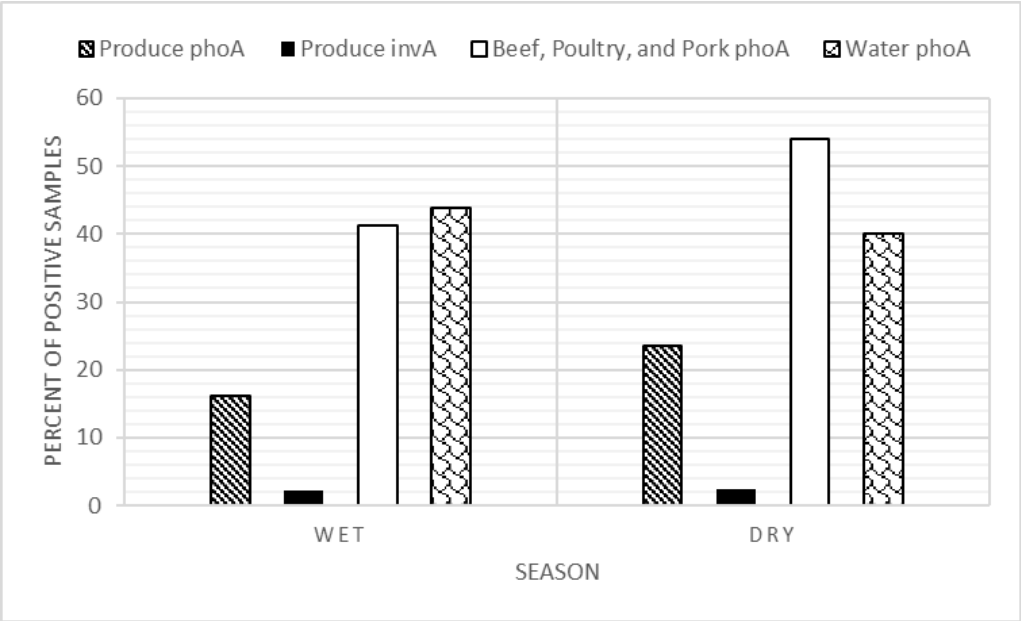

Figure 4: Total Samples containing the *phoA* and *invA* gene from different seasons in which samples were collected from the Chobe Region of Botswana, 2022. Produce sample collection: wet season (N=130), dry season (N=165). Beef, poultry, and pork sample collection: wet season (N=51), dry season (N=87).

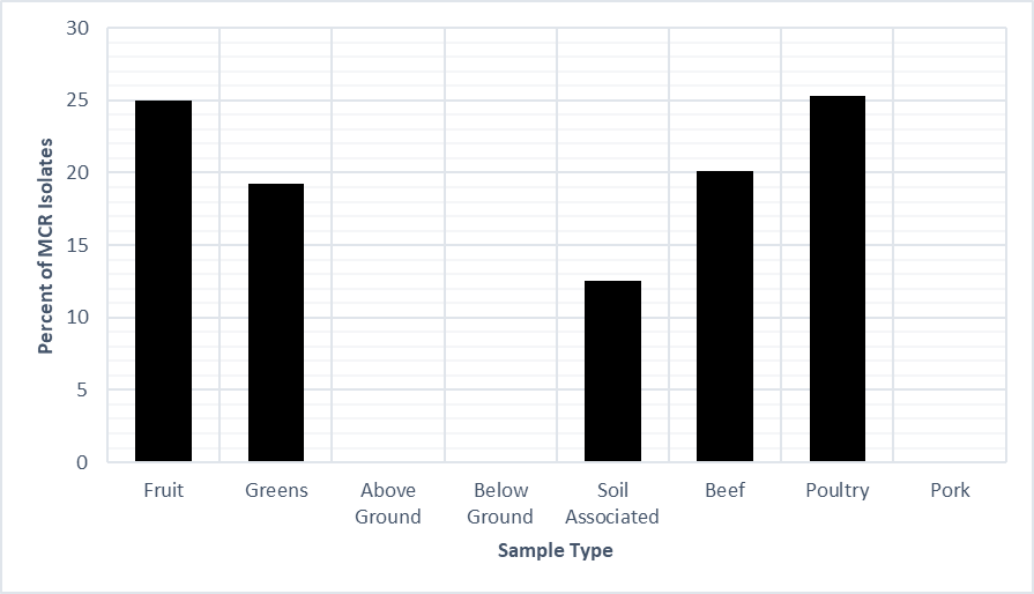

Figure 5: Total *E. coli* isolates that are MCR from different sample types collected from the Chobe Region of Botswana, 2022. Isolates screened: Fruit (N=24), Greens (N=26), Above Ground (N=16), Below Ground (N=14), Soil Associated (N=13), Beef (N=74), Poultry (N=79), Pork (N=4). No MCR *E. coli* isolates were found in the Above and Below Ground Groups.

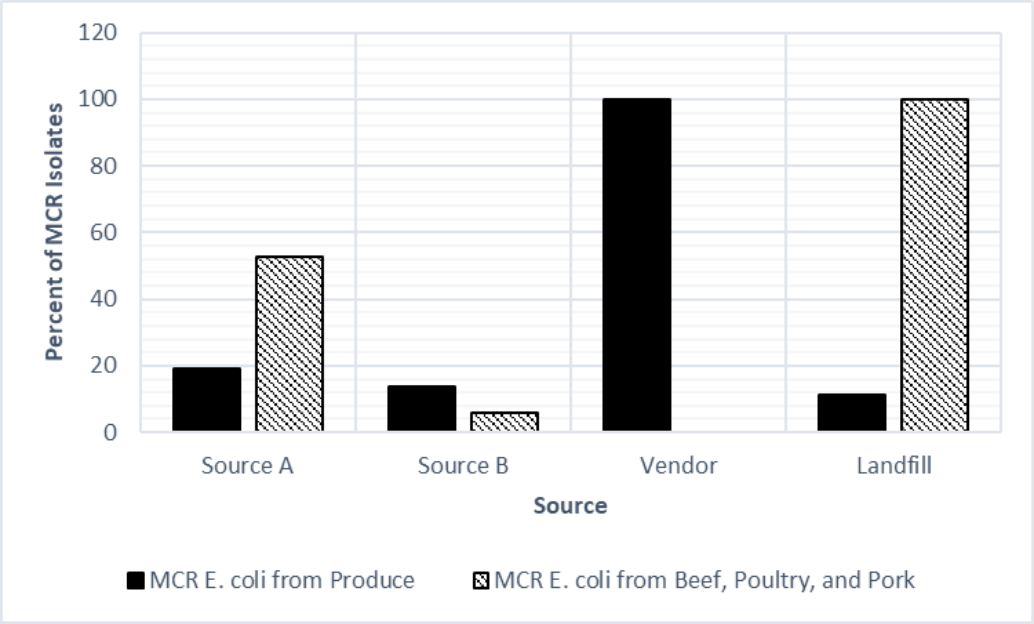

Figure 6: Total *E. coli* isolates that are MCR from different sources collected from the Chobe Region of Botswana, 2022. Produce isolates screened: Source A (N=26), Source B (N=22), Vendor (N=2), Landfill (N=53). Beef, poultry, and pork isolates screened: Source A (N=55), Source B (N=100), Vendor (N=0), Landfill (N=2).

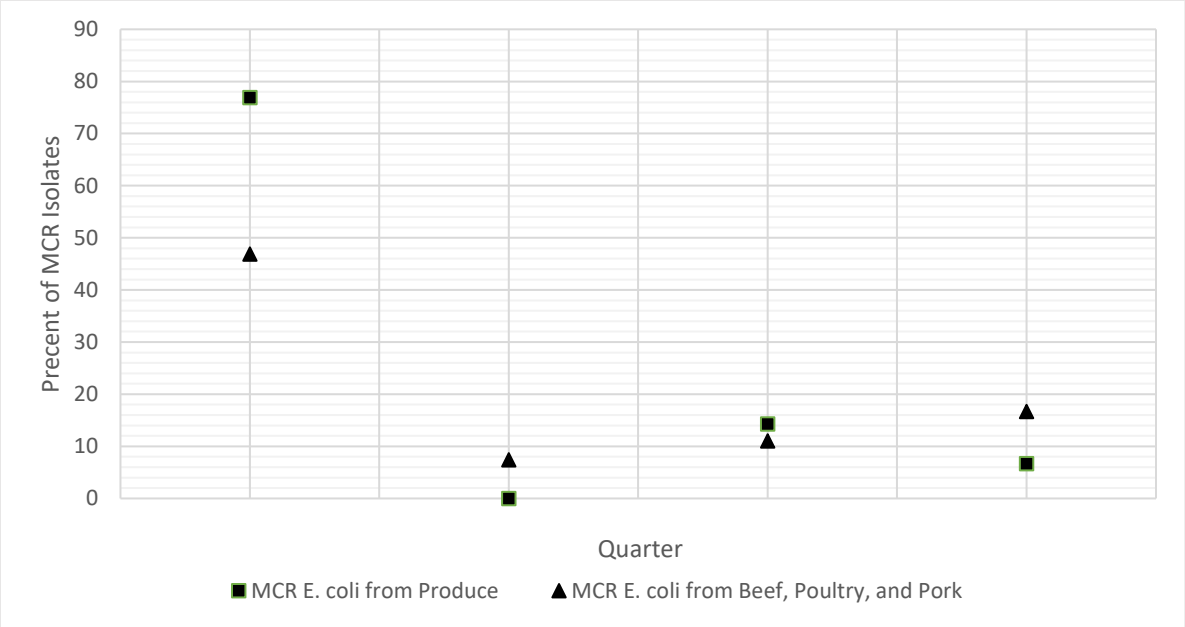

Figure 7: Total *E. coli* isolates that are MCR from different time periods (quarters) collected from the Chobe Region of Botswana, 2022. Number of produce isolates screened: January – March (N=13), April – June (N=40), July – September (N=35), October – December (N=15). Number of beef, poultry, and pork isolates screened: January – March (N=49), April – June (N=27), July – September (N=63), October – December (18).

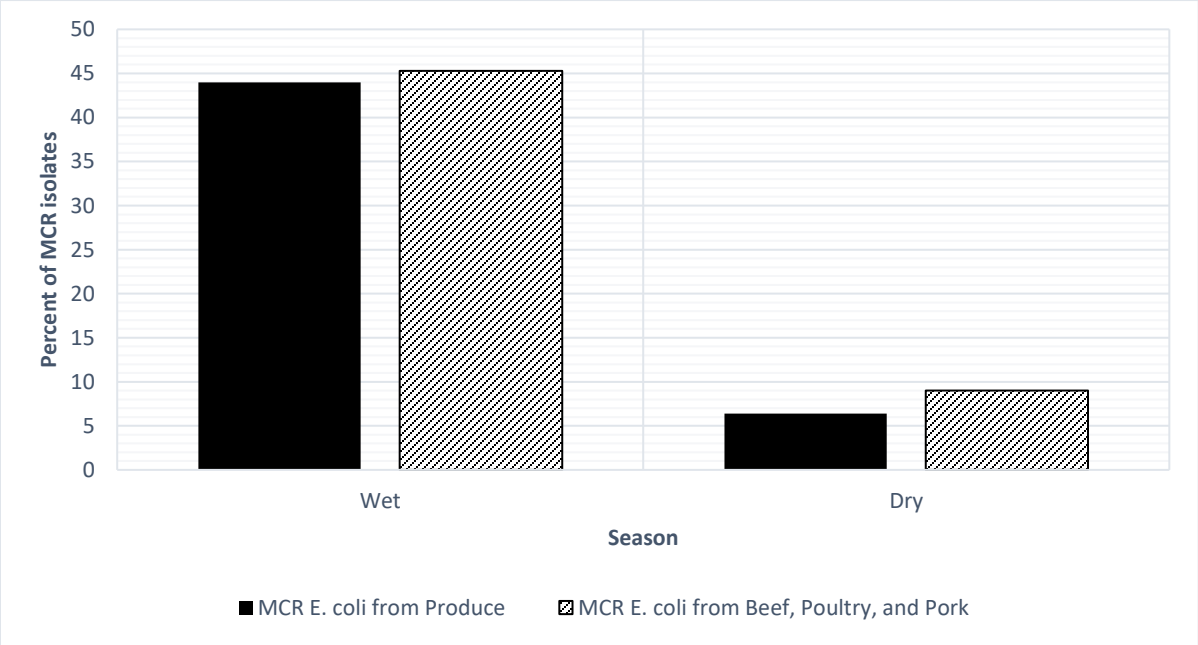

Figure 8: Total *E. coli* isolates that are MCR from different time periods (wet and dry seasons) collected from the Chobe Region of Botswana, 2022. Number of Produce Isolates Screened: wet season (N=25), dry season (N=78). Number of beef, poultry, and pork isolates screened: wet season (N=57), dry season (N=100).

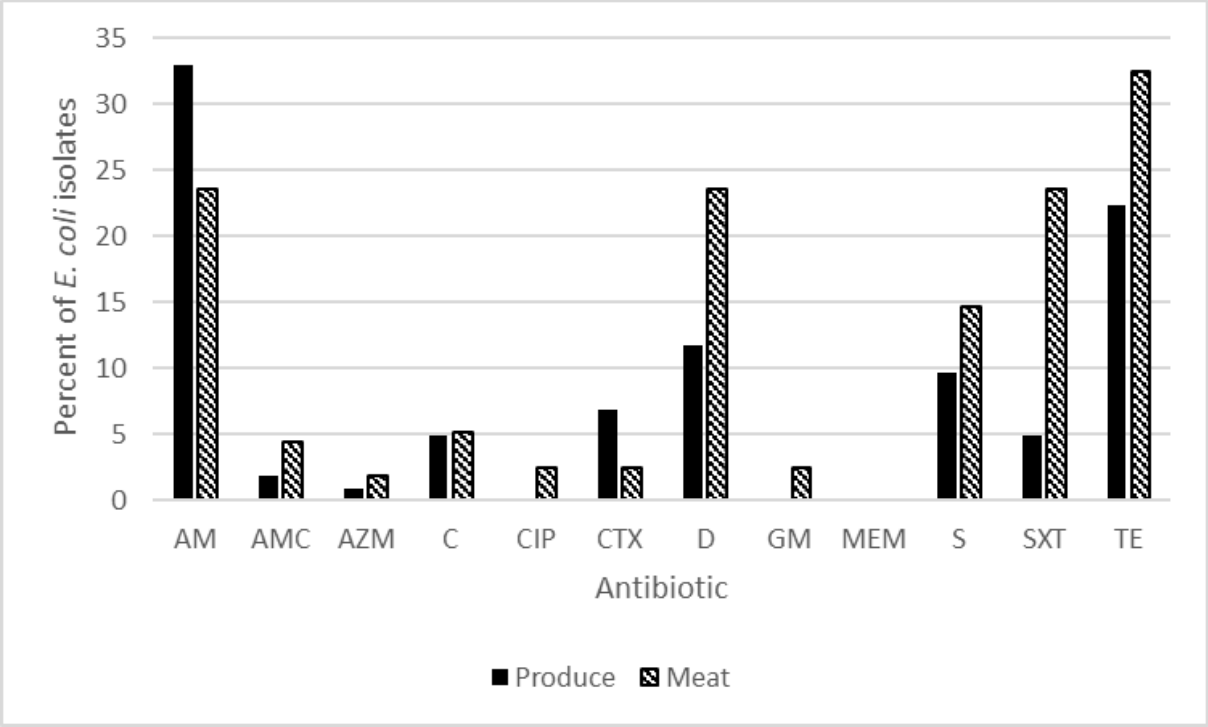

Figure 9: Total *E. coli* isolates by antibiotic from samples collected from Botswana, 2022. Produce isolates screened (N=103), beef, poultry, and pork isolates screened (N=157). No *E. coli* isolates from produce had resistance to gentamycin or meropenem. No *E. coli* isolates from beef, poultry, and pork were resistant to meropenem.
AM – Ampicillin: AMC – Amoxicillin: AZM – Azithromycin: C – Chloramphenicol: CIP – Ciprofloxacin: CTX – Cefotaxime: D – Doxycycline: GM – Gentamycin: MEM – Meropenem: S – Streptomycin: SXT – Trimethoprim/Sulfamethoxazole: TE - Tetracycline

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Chapter 5:
Conclusions and Future Research

This study identified that *Salmonella* and pathogenic *E. coli* are present in food in the Chobe region of Botswana and likely coming in through food. Pathogenic *E. coli* was present in greater percentages in the water than in food which is concerning since water has the potential to come into contact with food or be directly consumed. We also identified multi-class resistant *E. coli* isolates in produce, poultry, and water samples. These findings highlight the need for training in the area surrounding safe food handling, discretion in antibiotic use, and water sanitation including open defecation and restricting dumping human fecal waste in rivers.

To our knowledge, this is the first study to investigate prevalence and antibiotic resistance profiles of *Campylobacter, Salmonella,* and *E. coli* from food, food waste, and water samples from the Chobe region of Botswana over the span of a year. However, there are still several knowledge gaps to understand how these organisms circulate in the environment and the source of antibiotic resistance development. Genome sequencing of pathogenic and multi-class resistant isolates should be performed so they can better be traced through the environment to help develop plans to reduce further spread. Plasmids should be looked at in the multi-class resistance *E. coli* isolates to understand the transfer of antibiotic resistance traits in the Chobe region of Botswana. Antibiotic testing of the water samples will also help provide a broader picture of resistance. Better understanding the human fecal characteristics from the area can also provide another piece of the puzzle of how these bacteria move in the environment.

While limitations do exist, the findings of this thesis establish the prevalence of *E. coli* and *Salmonella* in the Chobe region of Botswana and their corresponding antibiotic resistance profiles. It also established spatial and temporal factors that influence the presence of *E. coli* and

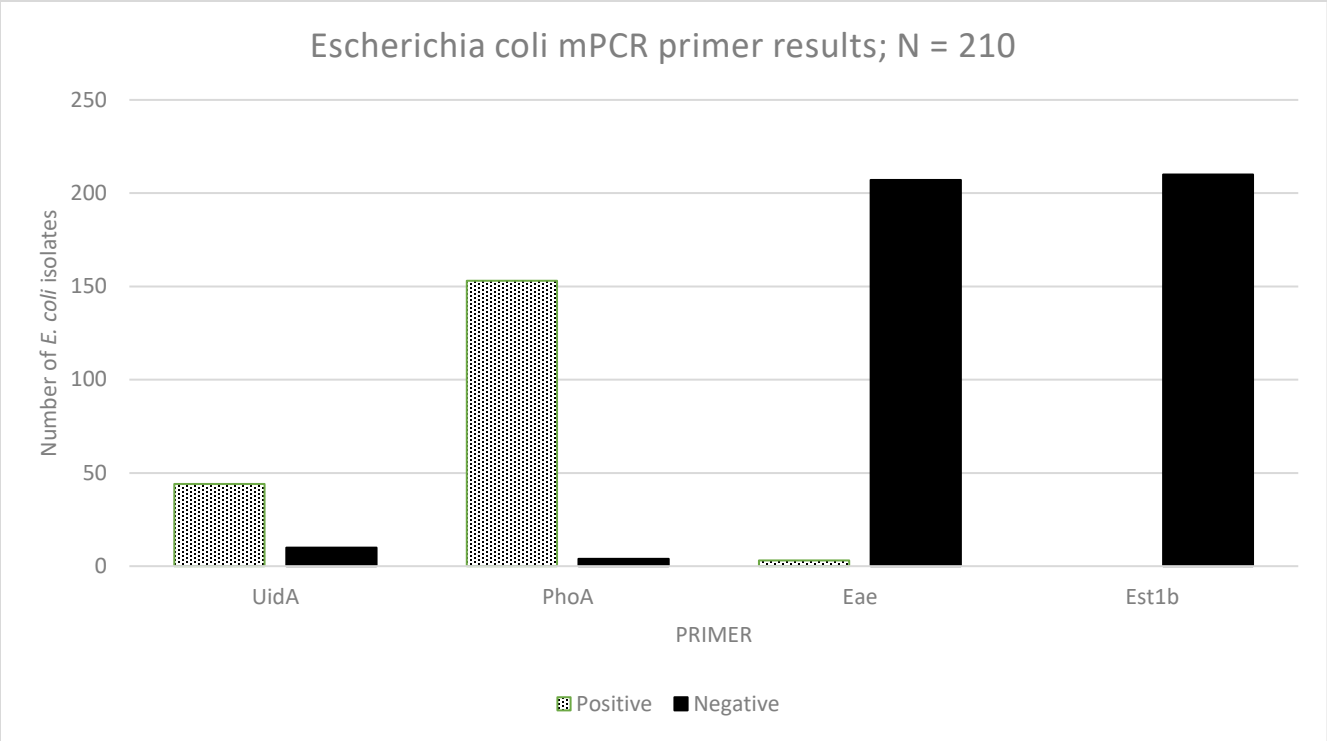
Salmonella and their antibiotic resistance profiles in food, food waste, and water samples. This project contributes to a larger grant to look at these bacterial agents in food, food waste, water, human fecal, and animal samples in the Chobe region of Botswana in order to establish a One Health approach to mitigating the threats these microorganisms can cause.

Appendix A

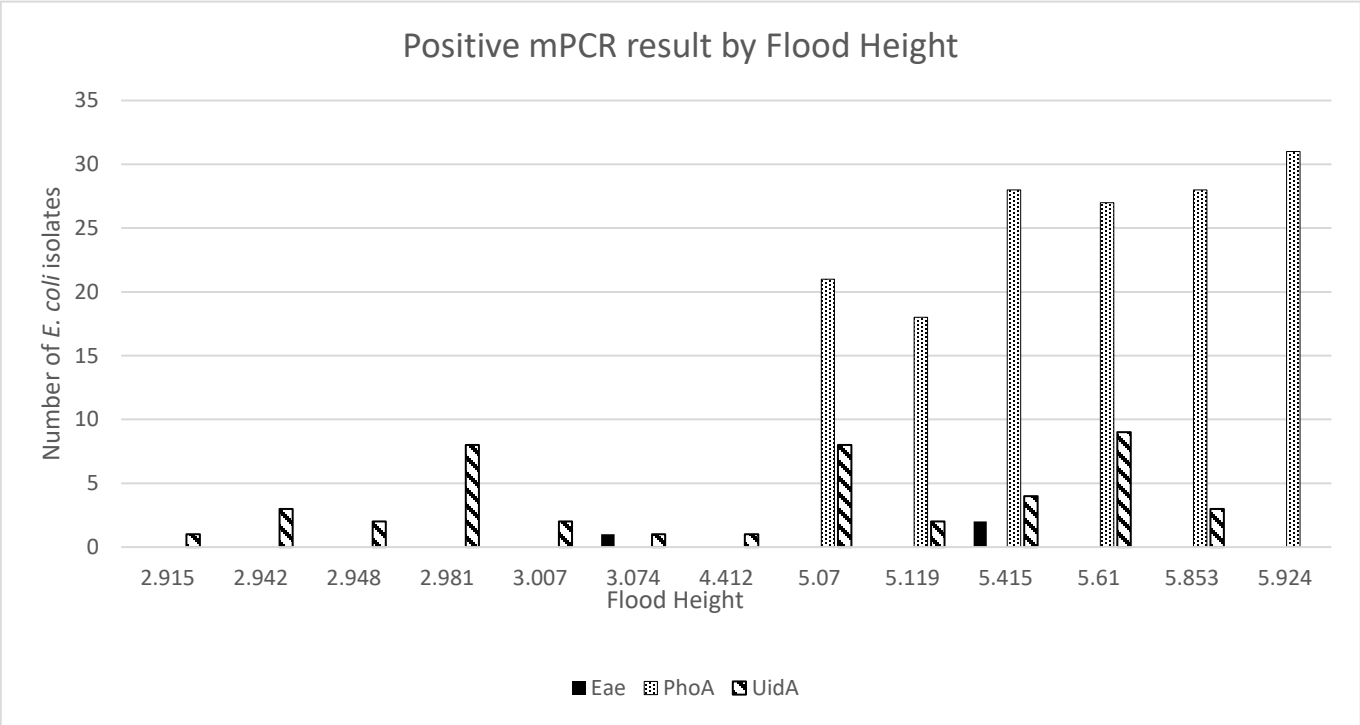
An additional 210 *E. coli* isolates were screened to test the accuracy of the *E. coli* mPCR protocol (Chapter 4 of this thesis) with environmental samples from the target region in this study. They were randomly selected from water isolates collected from the Chobe Region of Botswana from 2017-2018. The same methods were used as outlined above for water *E. coli* collection, screening, and isolation as well as Sanger sequencing. Initially, the *uidA* gene (n=54) was selected as the *E. coli* genera marker; however, it was discovered through sequencing these products that non-specific binding occurred resulting in false positives. It was switched for another gene *phoA* (n=157). One isolate did get tested with both primers, both of which were positive. The *uidA* primer yielded 44 positives and 10 negatives, the *phoA* primer yielded 153 positives and 4 negatives, *eae* yielded 3 positives and 207 negatives, and *est1b* yielded 0 positives and 210 negatives (Figure 1).

The isolates were also looked at by flood height (Figure 2). There appears to be an increase in *phoA* positives in the higher flood heights. However, it is important to note that when the protocol switched to the *phoA* primer, the high flood heights were prioritized to try and catch pathogenic *E. coli*. Out of the four *phoA* negatives, two occurred at a flood height of 5.07, and two occurred at a flood height of 5.119. No samples beneath a flood height of 5 were tested with *phoA.* It is interesting to note that three *eae* positives occurred. One was at 3.074 flood height and two at 5.415 height. The number of samples tested by flood height is listed in Table 1.

In summary, the amount of pathogenic *E. coli* isolates discovered through the *eae* gene from this study was low (n=3). These three isolates were confirmed by sequencing the PCR product. This is even when the second half of the isolates tested were picked from flood heights of 5 or higher in an attempt to find more pathogenic types.



Appendix, Figure 1: The total positive and negative results for each primer tested



Appendix, Figure 2: The total number of positives by primer and flood height

Appendix, Table 1: The number of *E. coli* isolates tested from water by flood height

Date	Flood Height	Number of Samples Tested
September 12, 2017	2.981	9
September 26, 2017	2.942	7
October 24, 2017	2.915	1
December 7, 2017	2.948	4
December 20, 2017	3.007	3
January 3, 2018	3.074	2
February 27, 2018	4.412	1
March 13, 2018	5.07	30
March 27, 2018	5.119	22
April 11, 2018	5.415	33
April 22, 2018	5.61	36
May 8, 2018	5.924	31
May 23, 2018	5.853	31