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SURVIVAL AND FITNESS OF *MCR-1*-HARBOURING *ESCHERICHIA COLI* ON SELECTED PRODUCE

by
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ABSTRACT

OF THE THESIS OF

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Antimicrobial resistance is a global public health concern. Colistin, also referred to as polymyxin E, is a last resort antibiotic used to treat complicated bacterial infections caused by Gram-negative bacteria when other antibiotics are proven ineffective. It was believed that colistin resistance was chromosomally mediated; however, *Liu et al*. have discovered the mobile colistin resistance gene in the year 2016. Following this discovery, the mobile colistin resistance gene, mcr-*1,* has been detected in several niches in more than 40 countries. Lebanon, a developing country, suffers a serious antimicrobial resistance issue with the dissemination and spread of the mobile colistin gene *mcr-1* in several matrices. Foodborne outbreaks related to consuming raw vegetables have been increasing with *Escherichia coli* reported as the most common pathogen behind these outbreaks. Multidrug resistanct *mcr-1*-harbouring *E.coli* have been detected in fresh produce in several countries. The sources of contamination of fresh produce include soil, irrigation water, manure, domestic and wild animals, insects, processing methods, and handling. Hence, the aim of this study is to quantify the survival and fitness of *mcr-1*-harbouring *Escherichia coli* on selected fresh produce, lettuce, and spinach. The study also aims to assess the potential changes of Antimicrobial resistance (AMR) profiles of these *E.coli* on fresh produce. The isolates used are from various sources, sewage water, poultry, river water, and pigeon fecal matter. Results of this study show that all used isolates survived on both lettuce and spinach for up to 72 hours at room temperature and up to 7 days at 4°C. The final bacterial load was evaluated to assess the survival potential of these isolates on the fresh produce samples used. The PCR analysis showed that all tested isolates from the last time points retained the mobile colistin resistance gene *mcr-1*. The disc diffusion method showed that the survived isolates of sewage source (WB3) and that of river water source (Waz3(1)) retrieved from the last time point did not exhibit any changes in their AMR profiles when compared to those of the original corresponding isolates. The other survived isolates showed minor changes in their AMR profiles when compared to their corresponding original isolates. This is the first study to evaluate the survival of multidrug-resistant *mcr-1*-harboring *Escherichia coli* in lettuce and spinach. The findings of this study highlight the urge to act to ban colistin use and limit its use to therapeutical purposes to stop the spread of colistin resistance genes from different matrices to humans.

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ABBREVIATIONS

TBX	Tryptone Bile X-glucuronide
WHO	World Health Organization
CLSI	Clinical and Laboratory standards Institute
PCR	Polymerase Chain Reaction
E. coli	*Escherichia coli*
mcr-1	*Mobile colistin resistance gene*
AMR	Antimicrobial resistance
MIC	Minimum inhibitory concentration
µL	Microliters
mL	Milliliters
Nm	Nanometer
rpm	revolutions per minute
bp	Base pairs
V	Volts
MDR	Multidrug resistant
CDC	Centers for Disease Control and Prevention
MRL	maximum residue limit
LPS	Lipopolysaccharide
ESBL	extended-spectrum beta-lactamase
ESC	expanded-spectrum cephalosporin
ACMSF	Advisory Committee on the Microbiological Safety of Food
RTE	ready-to-eat

DEPs	diarrheagenic E. coli pathotypes
LB	Luria-Bertani Broth
BPW	Buffered Peptone Water
OD	Optical Density
CFU	Colony Forming Unit

To
My Beloved Family

CHAPTER I

INTRODUCTION

A. The Problem of Antimicrobial Resistance

Antibiotics have had a major influence in the medical field and have saved millions of lives since their discovery. However, microorganisms have been rapidly developing resistance to antibiotics through natural mutations, and genetic material transfer. Further escalating the issue, the overuse and misuse of antibiotics by humans have significantly contributed to the emergence of antibiotic resistance bacteria [1]. In 2013, the US Centers for Disease Control and Prevention (CDC) claimed that humanity entered the post antibiotic era. Additionally, in 2014, the World Health Organization (WHO) classified Antimicrobial Resistance (AMR) as a global crisis. Antimicrobial resistance threatens the efficacy of antibiotics leading to the persistence of infections and thus increasing the spread rate of diseases among individuals. New resistance mechanisms are spreading globally leading to the inability to treat common infections and thus rendering them deadly. The emergence and spread of AMR are also complicating the routine medical surgeries and procedures and making them high risk processes. Moreover, the AMR crisis is placing a significant burden on health care systems since it is contributing to longer hospitalization periods requiring more intensive care. [2]. This issue is even more crucial in developing countries such as Lebanon due to many factors including poor infrastructure, weak antimicrobial stewardship, pollution, and refugee crisis [3].

1. Antibiotics Use and Consequences in Lebanon

In Lebanon, antibiotics are widely available in pharmacies without prescriptions. They are even available for online purchasing on websites. Several factors further complicate the AMR problem in the country such as i) the lack of awareness of the consumers and providers, ii) the overuse of antibiotics in humans, agriculture, and animals, iii) the use of antibiotics as growth promoters and prophylactic agents, iv) self-medication and lack of commitment to treatment timeline and doses. Furthermore, antibiotics may not be fully metabolized by the human body and large amounts may be excreted in the fecal matter or urine and enter wastewater treatment facilities. These intact antibiotics or active metabolites contribute to the spread of resistant microorganisms into the environment especially when effective sewage water management systems are lacking [3].

Antimicrobial usage in animals may leave edible tissues contaminated with antimicrobial residues which are toxic to consumers. Consumption of antibiotic residues even below the maximum residue limit (MRL) can cause the accumulation of these substances leading to several complications including allergies, disorder of intestinal flora, and emergence of resistant bacteria. In Lebanon, several studies investigated antibiotic residues in dairy and meat products. The results revealed high contamination of these foods with antibiotics [4,5,6].

2. Emergence of Antimicrobial Resistance in Environmental Settings in Lebanon

Water contaminated with resistant bacteria is a serious problem since it provides a route for AMR transmission to many niches and environmental settings. Lebanon-a developing country with poor infrastructure, and weak sewage water management systems suffers water contamination and spread of AMR from human, animal, and health care facilities sources [7].

A study investigated the contamination of irrigation water in two major agricultural areas in Lebanon; South Lebanon and the Beqaa Valley where a total of 27 irrigation water samples were collected in the year 2018. *E.coli* was detected in 44% of the samples. AMR analysis revealed that all of the isolates were Multidrug-resistant (MDR); resistant to at least four classes of antimicrobials. Resistance was observed against penicillin (100% of isolates), ampicillin (100%), amoxicillin/clavulanic acid (23%), cefepime (18%), cefotaxime (41%), cephalexin (73%), cefixime (32%), doripenem (5%), meropenem (5%), gentamicin (36%), kanamycin (64%), streptomycin (95%), tetracycline (100%), ciprofloxacin (36%), norfloxacin (45%), trimethoprim/sulfamethoxazole (SXT) (68%) and chloramphenicol (45%). The results demonstrate the contamination of irrigation water in Lebanon with MDR *E.coli* and thus the possibility of the spread of this contamination to other niches. [8]

Another study on the microbial contamination of seawater in Lebanon revealed that 45.5% of the 22 locations tested were contaminated with *E.coli*. Disk diffusion assay showed that all 16 *E.coli* isolates were multidrug-resistant (resistant to at least three antibiotic classes), exhibiting resistance to penicillin (100% of isolates), ampicillin (94%), amoxicillin/clavulanic acid (94%), cefepime (13%), cefotaxime (25%), cefalexin (75%), cefixime (19%), gentamicin (13%), kanamycin (38%), streptomycin (69%), tetracycline (81%), ciprofloxacin (63%), norfloxacin (13%), trimethoprim/sulfamethoxazole (69%) and chloramphenicol (63%) [7].

Moreover, a study published in 2020 investigated multidrug resistance in rainbow trout farms. Five *E. coli* were identified and confirmed multidrug-resistant exhibiting resistance to penicillin, ampicillin, amoxicillin + clavulanic acid, cephalexin, kanamycin, streptomycin, tetracycline, trimethoprim-sulfamethoxazole, and chloramphenicol [9].

Furthermore, a study on the molecular characterization as well as antimicrobial resistance profile of pathogenic bacteria of environmental source in Lebanon found that 94.7% of the tested samples were contaminated with either *Escherichia coli*, *Salmonella*, or both. Samples were collected from seawater, sediment, crab, and fresh water. Two out of the *E.coli* detected by PCR were O157:H7. Confirmed *E. coli* and *Salmonella* exhibited high rates of antimicrobial resistance. Given that a similarity in resistance profiles was observed between *Salmonella* and *E. coli* isolates, it was suggested that horizontal gene transfer may explain this phenomenon. Thus, not only is AMR emerging in environmental settings in Lebanon, but it is also spreading among different species occurring in nature [10].

A similar study investigated the presence and the molecular characterizations of *Streptococcus pneumoniae* in aquatic environments in Lebanon. The study focused on ground water, sea water, sediments, and crabs. Interestingly, crab samples were the most contaminated among the collected samples. Sea water was confirmed to contain *S. pneumoniae.* Moreover, twenty isolates from sea water were also confirmed resistant to vancomycin, gentamicin, and oxacillin. Other isolates were resistant to clindamycin and erythromycin. Tested freshwater samples contained isolates resistant to erythromycin, vancomycin, gentamicin, clindamycin, and oxacillin. Similar findings were observed in tested crab samples [11].

Another study conducted on the environmental settings in Lebanon targeted S*taphylococcus aureus* and *saprophyticus.* Representative samples were collected from sea water, fresh water, sediments, and crab along the coast of Lebanon. 51% of all tested bacteria were resistant to at least one of the antimicrobials used. The resistance pattern ranged from 45% in fresh water and 54.8% in sea water [12]. These studies illustrate the increasing antimicrobial resistance rates in Lebanese environment and water sources.

3. Emergence of Antimicrobial Resistance in Food Systems in Lebanon

Since most water sources in Lebanon were confirmed contaminated with antimicrobial resistant bacteria, further studies were conducted to investigate the AMR status in fresh produce. A study conducted in the Beqaa valley revealed 60 *E. coli* isolates from vegetables collected from restaurants, markets, farms, and crop washing areas. All isolates were resistant to streptomycin and cephalothin. Resistance to ampicillin, tetracycline, gentamycin and colistin sulphate was detected in 78%, 42%, 22% and 3% of the isolates, respectively. Overall, 60% of the isolates were multidrug-resistant which is a high number compared to other studies [13].

Dairy products are also susceptible to contamination due to milk composition and nutrients [14,15]. The Lebanese diet highly depends on many dairy products (such as yogurt, labneh, milk, cheese, kishk and shankleesh) [16]. Products made with unpasteurized milk are more frequently contaminated [14]. In addition, raw milk used for handmade products is sometimes heated for a few minutes only at temperatures that cannot kill all bacteria [15]. To investigate the AMR problem in dairy products, a series of 5 studies was conducted on 3 traditionally consumed Lebanese dairy products from Beqaa region since this area is known to be a major source of dairy products in Lebanon. Most products are prepared using traditional techniques with minimal hygiene practices [15]. One-hundred sixty-four samples (83 kishk, 45 baladi cheese and 36 shankleesh) were collected from houses, markets, and small farms, and were all available for sale

[14,15]. Each study focused on one specific bacterial type and analyzed the antibiotic resistance profiles.

The findings illustrated the presence of *Brucella abortus* in different ready-to-eat dairy products. Six isolates were confirmed and tested for antimicrobial susceptibility. Multidrug resistance patterns were observed with all isolates resistant to at least one of the used antibiotics; streptomycin and ciprofloxacin, and gentamicin [17].

The studies also confirmed that 94 *E. coli* isolated from 164 samples of dairy products of which 38.3% belonged to pathogenic species. Antimicrobial susceptibility tests revealed that all isolated *E.coli* were resistant to at least one of the ten tested antibiotics, 84% of which were resistant to tetracyclines. [14]

Furthermore, the samples were tested for the presence of *Listeria monocytogenes*. All isolates were resistant to at least one antibiotic, and the highest resistance rates were to oxacillin, penicillin, and ampicillin. Surprisingly, about 26% of the isolates were vancomycin resistant which is high comparing to other studies [18]. *Yersinia enterocolitica* was also isolated from the same samples and found highly resistant to streptomycin (87.5%). The isolates were also highly resistant to most antibiotics, which is odd knowing that *Yersinia* does not develop resistance easily as it was confirmed by previous studies [19].

Moreover, same dairy products samples were found to contain *Staphylococcus aureus* and *Staphylococcus saprophyticus.* 45 isolates were confirmed resistant to at least one antibiotic, 61% of which exhibited resistance to all 6 tested antibiotics with the highest resistance to oxacillin and clindamycin followed by methicillin, teicoplanin, vancomycin and gentamicin. [15]. These results indicate high levels of antimicrobial resistance prevalence in some of the most consumed dairy products in Lebanon which is alarming given the lack of AMR control mechanisms in the country.

Other studies investigated the presence and antimicrobial resistance profile of isolates from meat-based foods in Lebanon. Two of the famous Lebanese foods were included; Lahm-bi-Ajeen (meat pies) and Shawarma. The isolates were identified and tested for resistance patterns to a variety of commonly used antimicrobials. Results indicated contamination of the tested foods with *Salmonella paratyphi* (serogroup A) and Shiga Toxin producing *E.coli*. The confirmed isolates were highly resistant to at least one of the tested antimicrobials. Almost 69% of *E.coli* and 77.8% of *Salmonella* species were antimicrobial resistant. Moreover, all tested *Salmonella* species were resistant to clindamycin, oxacillin, vancomycin, teicoplanin, and erythromycin whereas all *E.coli* isolates were resistant to trimethoprim and teicoplanin. These findings highlight the presence of pathogenic bacterial strains that are highly resistant to different antimicrobials in two of the commonly consumed meat-based foods in Lebanon [20].

Another study examined 50 minced raw meat samples that were collected from different stores and butcheries in Beirut, Lebanon. A hundred twenty *E.coli* were isolated and tested for antimicrobial resistance profile. 98% of the meat samples contained fecal coliforms and 76% yielded *E. coli* above the accepted microbial level. All *E. coli* isolates were resistant to more than one antibiotic tested whereas 35 % of the isolates were multidrug resistant. The findings emphasized the issue of antimicrobial resistance in one of the most common consumed foods in Lebanon [21].

Moreover, a study identified 51 *Campylobacter coli* isolates from two slaughterhouses in Lebanon. Collected samples were both chicken caeca taken during the evisceration and whole poultry carcass. Antimicrobial susceptibility testing was performed involving a variety of β-lactam agents and tetracycline. The AMR profile of these isolates revealed that all isolates were resistant to cephalothin and aztreonam. Isolates were also resistant to cefamandole and cefoxitin (98%) tetracycline (94%), ampicillin (49%), amoxicillin (47%), piperacillin (45%), carbenicillin (37%), ticarcillin (20%), ceftazidime (18%), cefotaxime (8%) and amoxicillin-clavulanic acid (2%) [22].

Another study investigated the presence of multi-drug resistant Gram-negative Bacilli in poultry farms across Lebanon through collecting 981 fecal swabs. Two hundred thirty-five strains were isolated and identified as *E. coli* (92%), *Klebsiella Pneumoniae* (3%), *Proteus mirabilis* (1%), and *Enterobacter cloacae* (1%). "The phenotypic tests revealed that 43.5, 28.5, and 20.5% of the strains were ampC, ESBL, and ampC/ESBL producers respectively. The putative TEM gene was detected in 83% of the isolates, SHV in 20%, CTX-M in 53% and CMY ampC b-lactamase gene in 65%." These findings confirm that chicken farms across Lebanon present a reservoir of Multi-Drug Resistant Gram-negative bacilli [23].

Another study investigated the emergence of antimicrobial resistant *E.coli* in Lebanese poultry farms. Ninety-three fecal samples were collected from three major Lebanese broiler chicken farms. The results showed that 97% of the tested samples yielded *E.coli* colonies 90 of which were isolated and further purified. Disk diffusion essay showed that all tested isolates were multidrug resistant (Resistant to 4 or more classes of antimicrobials). The isolates exhibited resistant to penicillin (100% of isolates), ampicillin (100%), amoxicillin-clavulanic acid (56%), cefepime (25%), cefotaxime (70%), cephalexin (94%), cefixime (76%), doripenem (1%), imipenem (1%), meropenem (2%), gentamicin (61%), kanamycin (76%), streptomycin (80%), tetracycline (89%), ciprofloxacin (91%), norfloxacin (68%), trimethoprim-sulfamethoxazole (84%), and chloramphenicol (95%). [24]

Another study targeted Rainbow Trout in Lebanon where 5 *E.coli* isolates were assessed for their antimicrobial susceptibility. All isolates were multidrug-resistant and disk diffusion assay showed that both F1 isolates (F1I1 and F1I2) were resistant to penicillin (PEN), ampicillin (AMP), amoxicillin/clavulanate (AMC), cephalexin (LEX), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), tetracycline (TET), trimethoprim/sulfamethoxazole (SXT), and chloramphenicol (CHL). Moreover, all three F2 isolates (F2I1, F2I2, and F2I3) exhibited resistance to PEN, AMP, AMC, LEX, KAN, STR, TET, ciprofloxacin (CIP), norfloxacin (NOR), SXT, and CHL. [9]

B. Colistin Resistance and *mcr-1*

Colistin, also known as Polymyxin E, is a polypeptide antibiotic that belongs to the polymyxin family of drugs. It was isolated from the soil bacterium *Paenibacillus polymyxa subsp colistinus* in Japan in 1947. It was first used as an intravenous formulation in 1950 and became available for clinical use in 1959 upon getting approved by the US Food and Drug Administration (FDA) [25]. The use of colistin to treat bacterial infections was largely abandoned in the 1970s because of its neurotoxicity and nephrotoxicity. However, colistin has been reintroduced as a last resort antibiotic in recent years as drug-resistant infections have emerged. Moreover, plasmid-mediated colistin resistance conferred by the mobile colistin resistance gene (*mcr-1*) was identified in the late 2015 and the prevalence of colistin resistance has thus become a public health concern. Before the discovery of the *mcr-1* gene, resistance to colistin has been solely attributed to mutational and regulatory changes by chromosomal genes. *Mcr-1* gene was first described in a plasmid carried by an *E.coli* isolated in China in 2011. [26]

Lipopolysaccharide (LPS), more exactly lipid A is the initial target site of colistin. Lipid A is located in the outer membrane playing a significant role in cell permeability. The electrostatic interaction between the positively charged region of colistin and the negatively charged phosphate groups of lipid A, replace the calcium and magnesium ions previously linked to the phosphate group. The replacement of theses ions destabilizes lipid A and increases the outer cellular membrane permeability causing the entry of colistin and thus the eventual death of the bacteria. Another antibacterial mechanism of colistin is the inhibition of type II NADH-quinone oxidoreductase (NDH-2) which is a crucial respiratory enzyme in the bacterial cell membrane. The overuse of colistin has resulted in the emergence of colistin resistance worldwide. Resistance to colistin is achieved through the modification of LPS, and thus reducing or eliminating affinity for colistin. This mechanism may differ among species although it is common for Gram-negative bacteria. Changes of lipid A through the addition of positively charged residues such as phosphoethanolamine (PEtn) and/or 4-amino-4-deoxy-L-arabinose (L-Ara4N) decrease the overall negative charge of LPS, leading to a weaker electrostatic interaction with the positive charge of colistin which inhibits cell lysis. [27]

Mobile colistin resistance presents a significant public health concern as it can spread through horizontal transfer and may imply a lower fitness cost. *Mcr-1* has been identified in several countries and has been observed on plasmids that contain other antimicrobial resistance genes such as extended-spectrum β-lactamases and carbapenemases. *Mcr-1* has been observed on a variety of plasmid types including IncI2, IncHI2, and IncX4. The prevalence of *mcr-1* has been found high in some environmental settings, including the Haihe River in China, fecal samples from otherwise healthy individuals, and in public urban beaches water in Brazil. [26]

Several studies have documented the dissemination and spread of *mcr-1* in several matrices. To evaluate the potential risk of contamination of food with antibiotic-resistant bacteria, a study conducted in Egypt found that 78% of the raw beef collected samples and 53% of ready-to-eat beef products samples were contaminated with *Escherichia coli*. Eight *E.coli* of the 210 isolates were resistant to colistin and harbored *mcr-1* gene. Three *E.coli* of the colistin-resistant isolates harbored both *mcr-1* and extended-spectrum beta-lactamase (ESBL) genes comprising a significant public health concern. Colistin is frequently used in livestock production in Egypt. Thus, the results of the study suggest that colistin-resistant pathogenic *E.coli* are transferred from food producing animals to humans via meat and meat-based products. [28]

A study published in 2020 reported the detection of the mobile colistin resistance gene *mcr-1* in multidrug resistant *E.coli* isolate recovered from lettuce produced and marketed in Portugal obtained at a retail store in the region of Lisbon and Tagus Valley. The *mcr-1* positive isolate revealed a non-wild-type phenotype to colistin (MIC 16 mg/L). This study emphasizes the severity of colistin resistance especially that *mcr-1* gene was detected in lettuce that is consumed raw with no further cooking or processing required. [29]

Several studies have been conducted in China to investigate contamination of fresh produce with *mcr-1*-harboring bacteria. One of the studies, where 538 fresh vegetable samples were collected from 53 supermarkets from 9 provinces in the country, 23 *mcr-1* positive *E.coli* were isolated from 19 fresh produce samples. [30]

Similarly, a study conducted in Switzerland targeted 60 extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* isolated from 42 imported vegetable samples (from Thailand and Vietnam). The study reported the detection of *mcr-1* gene in 2 out the 60 isolates that were all confirmed as *E.coli*. [31]

1. Colistin Resistance and mcr-1 in Lebanon

Several studies have been conducted in Lebanon to evaluate the presence of the mobile colistin resistance gene (*mcr-1*) in different matrices. One of the most recent studies investigated resistance to colistin as well as other antimicrobials in Gram-negative bacteria isolated from patients in North-Lebanon. Eight colistin-resistant *Escherichia coli* were isolated. All *E.coli* isolates exhibited minimum inhibitory concentration (MICs) greater than 2mg/L. [32]

Another study investigated the presence and spread of expanded-spectrum cephalosporin (ESC)-resistant *Escherichia coli* in healthy broilers in Lebanon. Two-hundred eighty rectal swabs were screened from 56 farms for the presence of ESC-resistant *E. coli*. *E.coli* (n=102) representative isolates were tested. Out of the 56 farms concerned, 52 housed broilers carrying ESC-resistant *E. coli* isolates. Nineteen out of the 102 isolates from 11 unrelated sequence types carried the mobile resistance gene *mcr-1* as well as other antimicrobial-resistance genes. The results of this study emphasize the alarming prevalence of Multi-drug resistant *E.coli* resistant to critically important antibiotics in broilers in Lebanon and advocate the urge to control excessive use of antibiotics in food-producing animals. [33]

Furthermore, a study investigated the occurrence of resistance genes including *mcr-1* gene in the community. For this purpose, 72 fecal samples were randomly collected from toddlers diapers from reputable daycares at five major locations across Lebanon. Thirteen samples yielded *E.coli* and 24 *E.coli* samples were purified and further processed. All isolates were confirmed to harbor *mcr-1* gene and the minimum inhibitory concentration MIC of colistin against the isolates ranged between 4 and 32 µg/ml. [34]

Moreover, two multi-drug resistant *E.coli* were isolated from drinking reservoirs and wells water used by refugee camps in Lebanon. The isolates exhibited resistance to important antibiotics including colistin and harbored the mobile colistin gene *mcr-1-1*. [35]

A study conducted in 2019 targeted the Lebanese irrigation water to detect the emergence of resistance to colistin; a last resort antibiotic to treat Gram negative bacterial infections. A total of 27 water samples were collected from two main Lebanese agricultural regions. Twenty-two *E. coli* were detected and confirmed to harbor the mobile colistin resistance gene *mcr-1*. Additionally, an antibiotic susceptibility test showed that all isolates were multidrug-resistant [8].

Following the prevalence of *mcr-1* positive, multidrug-resistant *E. coli* in irrigation water in Lebanon, a study focused on the spread of antimicrobial resistance in the Mediterranean Sea on the coastline of Lebanon. Twenty-two water samples were collected covering the country from North to South. Ten samples contained *E. coli* and 16 *mcr-1* positive *isolates* were confirmed. [7]

In another study, 93 fecal swabs were collected from three Lebanese broiler chicken farms between 2017 and 2018. Ninety *E. coli* were isolated and screened for *mcr-1* presence. All isolates were 16S rRNA positive and 88 of them harbored *mcr-1* (≈98%). All *mcr-1* positive isolates were multidrug resistant [24].

C. Fresh Produce Contamination with *E. coli*

Lettuce, spinach, and other raw vegetables are incorporated into many dishes including ready-to-eat salads and sauces [36]. These foods contribute a substantial nutritional value to the human's diet. In the USA, an increase of 25% in the average amount of consumed fresh produce per person has been reported over the last 30 years [37]. Conversely, foodborne outbreaks related to consuming raw vegetables have been increasing with *Escherichia coli* reported as the most common pathogen behind these outbreaks. Pathogenic *E. coli* can cause diarrhea, hemolytic uremic syndrome, hemorrhagic colitis, and other incidences in humans. Fresh produce can be contaminated with *E. coli* at any stage from preharvest to postharvest. This microorganism can survive under many environmental conditions due to various mechanisms including adhesion to surfaces and internalization in fresh produce. Thus, limiting the effectiveness of the available processing and sanitizing methods employed in the food industry [38].

Leafy greens including spinach, lettuce, and fresh herbs are the most frequently reported vegetables associated with bacterial infections [39]. In the US, the Food Safety Project reported at least 713 produce-related outbreaks associated with foodborne disease, 12% of which involved fresh vegetables and fruits [40]. According to a report published in 2011 by the Advisory Committee on the Microbiological Safety of Food (ACMSF), 531 cases of reported illness and 1 death in the UK were related to consuming vegetables and fruits between the years of 2008 and 2010 [41].

The source of contamination of fresh produce may be the manure, sewage, soil, surface water, or wildlife. This contamination may also occur during soaking, washing, slicing, packaging, and food preparation with the highest contamination taking place in the field, during initial processing, and in the kitchen. When it comes to leafy greens, the most common source of contamination is contaminated water [42]. According to the Centers for Disease Control and Prevention (CDC), the main source of fecal contamination of leafy vegetables in organic food production particularly in the harvest phase is the use of animal manure [43].

Wildlife and domestic animals are another potential source of pathogenic bacteria mainly for lettuce and leafy greens at the preharvest levels in the coast of California and in Yuma, Arizona [44]. Insects may also represent a source of contamination for plants such as flies that have been proven to transfer *E. coli* to fruits and plant leaves [45].

Furthermore, vegetables could get contaminated if they are prepared using implements that are not clean in restaurants or home kitchens. According to Lynch et al., contamination of vegetables with pathogens such as *E. coli* may occur through cross contamination due to poor hygienic practices when raw meat or poultry are being prepared [46].The presence of *E. coli* in produce is important as the vegetables are used to prepare fresh foods and low doses of infection can cause intestinal disease [38].

1. Internalization of Microorganisms into Fruits and Vegetables

Rich data is available confirming the internalization of human and plant pathogens into vegetables and fruits, mechanisms employed by these pathogens to enter fresh produce, and different produce prone to microbial contamination. A variety of pathways by which microorganisms enter produce exist due to the natural structure of some produce. For instance, bacteria can enter plant leaves through stomata and enter fruits through the stem, stem scar, or calyx. A study conducted in this regard found that a suspension of *E.coli* O157:H7 was absorbed by the stomata and cut surfaces of lettuce leaves. Moreover, birds, insects, and dust can be the source of contamination by acting as vectors particularly if the vegetables and fruits have injuries. Houseflies for instance were found to contain 100 pathogens of different species, 65 of which were confirmed to be transmitted. [47]

Several studies have confirmed that the human pathogen *E. coli* O157:H7 can be carried by birds and insects. A field study found *E. coli* O157:H7 contamination of wild houseflies on cattle farm that persisted the entire study period; 3 months. The suggested source of contamination was cow manure that the flies were frequenting. Another controlled study focused on the possibility for *E. coli* O157:H7 transmission to injured apples by fruit flies. The study results demonstrated that injured apples had high incidences of *E. coli* O157:H7 contamination when flies inoculated with pathogen contacted the apples. [47]

2. Survival of E. coli on Vegetables

For a pathogen that has been internalized withing vegetables or fruits to become a public health hazard, it must be capable of surviving until it reaches the consumer. Survival of pathogens depends on several factors such as the physical and chemical properties of fruits and vegetables, the processes applied during the preharvest phase, and the consumer handling. [47] The consumption of ready-to-eat (RTE) salads has been increasing worldwide. Thus, an increase in the number of outbreaks associated with food-borne pathogens, including diarrheagenic *E. coli* pathotypes (DEPs) linked to consuming ready-to-eat salads has been reported. In developing regions of the world, fresh produce is continuously irrigated with sewage water that is untreated. A study conducted in Hidalgo, Mexico, aimed to evaluate the microbiological quality and prevalence of DEPs in salads composed of raw vegetables purchased from restaurants at Pachuca-City where most of the locally produced and consumed vegetables are irrigated with sewage water that is untreated. A total of six restaurants were targeted where 130 salads were purchased. The restaurants ranged from national chain restaurants to local small restaurants. Each collected sample was tested to check for the presence of fecal coliforms and *E. coli*. Among the 130 salad samples, 129 (99%) of the samples were contaminated with fecal coliforms, 85% of which were contaminated with *E. coli*. The positive samples for fecal coliforms and *E. coli* were indifferent among all types of restaurants. Regardless of the overall hygienic status of the restaurants, most ready-to-eat salads had poor microbiological quality with some samples harboring diarrheagenic *E. coli* pathotypes that were associated with disease in Mexico [37]

In Lebanon, there is a notable lack of data when it comes to survival of pathogens on fresh produce. Thus, further research needs to be conducted in this regard given the importance of the issue especially that raw vegetables are incorporated in several meals in the country.

3. Survival in the Phyllosphere

Studies have been conducted to evaluate the fitness of enteropathogens on the leaf surface (phylloplane) of fresh produce. The ability of a pathogen to persist on the leaf surface results in increased chances of the infectious dose remaining at consumption. Taking for instance the study conducted on the survival of *E.coli* O157:H7 isolated from bovine fecal matter and inoculated in lettuce. The study demonstrated the ability to retrieve these isolates from lettuce samples up to 15 days after inoculation suggesting the ability of transient enteropathogens to be incorporated into the phylloplane biofilms. Another study suggested that bacterial gene expression, motility, and production of extracellular materials are not required for the primary attachment but may be vital in the colonization and survival on the leaf surface. Further research is required to investigate the factors capable of limiting the survival of enteropathogens in the field. This includes intervention strategies to limit the effects of bacterial persistence on the plant surface, in water and organic fertilizers, and irrigation water [48].

In Lebanon, multidrug resistant *mcr-1*-harbouring *Escherichia coli* has been detected in several niches including various water sources, fecal matter, aquaculture, food producing animals, and humans. The overuse of antibiotics and the ineffective wastewater management systems are some of the factors that have contributed to the spread of antimicrobial resistance from different sources to humans. However, no data is available on the survival of these microorganisms in food products. Thus, the objectives of this thesis is to quantify the survival of *mcr-1*-positive *E. coli* from different origins (sewage water, chicken, river water, and pigeon fecal matter) on green fresh produce; lettuce and spinach and to assess potential changes of AMR profiles of *mcr-1* positive *E.coli* on fresh produce . Thus, the hypothesis of this study is that *mcr-1* carriage adversely impacts the survival and fitness of *E. coli* on fresh produce.

CHAPTER II

MATERIALS AND METHODS

A. Preparation of Samples

Fresh produce, including lettuce (*Lactuca sativa*) and spinach (*Spinacia oleracea*), were inoculated with *mcr-1* carrying *E. coli* isolated from different matrices in Lebanon including sewage water, poultry, river water, and pigeon fecal matter. The produce samples were divided into pieces of equal surface areas, (5cmx5cm) washed twice with distilled water, and sterilized with 70% alcohol then rinsed twice with autoclaved deionized water. The samples were then allowed to dry in a sterile environment (biosafety cabinet) before the inoculation step. Control samples that were not inoculated with the test isolates were prepared in the same manner.

Figure 1. Lettuce sample prepared for inoculation

B. Inoculation of Produce

Previously stocked *E. coli* isolates were cultured overnight in Luria-Bertani (LB) broth supplemented with 4 µg/ml colistin. The overnight cultures were then diluted (1/10), supplemented with 4 µg/ml colistin, and allowed to grow to exponential phase for 2 hours. After that, the cultures were pelted and washed. Specifically, cultures were centrifuged for 5 minutes at maximum speed (13000 rpm) then the supernatant was discarded. The resulting pellet was then resuspended in 1 ml Buffered Peptone Water (BPW), vortexed, and recentrifuged at the same conditions. A second washing step was conducted, and the resulting pellet was resuspended in 1 ml BPW. The inoculum was then prepared so that the OD equals to 0.05 at wavelength of 600 nm. The prepared produce samples were then inoculated with 30 µl of the prepared inoculum distributed into 3 10 µl drops on produce samples. The resulting spots were then allowed to dry.

Figure 2. Lettuce sample inoculated with 3 (10 µl) drops of bacterial inoculum

C. Storage of Inoculated Samples and Quantification of *E. coli* Survival and Persistence of *mcr-1*

The inoculated produce pieces as well as control samples that were kept un-inoculated were placed in a stomaching bag and placed at room temperature. Replicate bags were placed in the 4 °C to evaluate survival of the isolates under refrigeration. At different time points (With 6, 12 and 24 hour-intervals for samples at room temperature, and 24 hour-intervals for samples at 4°C), the produce pieces were weighed and suspended in the corresponding volumes of BPW. Homogenization was then conducted through stomaching for 5 minutes. Different serial dilutions (1/10) were prepared and 100 µl were spread on TBX Agar media supplemented with 4 µg/ml colistin. The plates were then incubated at 37 °C for 24 hours to count the colonies. The experiments were done in duplicates. The colonies from the last time point were then heavy streaked on Tryptone Bile X-glucuronide (TBX) Agar media supplemented with 4 µg/ml colistin and incubated at 37 °C for 24 hours. Un-inoculated sterile vegetable samples were also incubated in similar conditions (control) with enumeration of bacterial count at similar time intervals. The mean CFU/g was used to plot the CFU/g vs time graphs. Stocks were prepared and stored in 1 ml LB broth with 0.5 ml 80% glycerol and stored at -80°C for further analysis.

D. Polymerase Chain Reaction-Based Analysis

Genomic DNA was extracted from bacterial colonies using boil preparation. Bacterial colonies were suspended in 100 µl DNase free water and placed in a water bath at 95°C for 13 minutes. The tubes were then centrifuged for 2 minutes at 13000 rpm. The supernatant was collected and transferred into sterile tubes. The tubes were then placed at -20°C for further analysis. The extracted genomic DNA was used as a template for the polymerase chain reaction analysis.

1. Detection of Mobile colistin Resistance Gene Using PCR

The extracted genomic DNA was used to screen for the presence of the mobile colistin resistance gene (*mcr-1*). The reaction was carried using two specific primers, the forward CLR5 primer (CLR5-F 5′-CGGTCAGTCCGTTTGTTC-3′) and the reverse CLR5-R primer (5′-CTTGGTCGGTCTGTA GGG-3’). The reaction mixture was prepared in sterile pcr tubes by adding 3 µl of genomic DNA to a mixture consisting of 12 µl of DNase free water, 4µl of Master Mix (5x FIREPol® Master Mix Ready to Load), and 0.5µl of each of the two primers, forward and reverse. The polymerase chain reaction consisted of 38 cycles, each cycle includes a denaturation step at 95°C for 1 minute, followed by annealing of the primers at 56°C for 45 seconds and finally an extension step at 72°C for 1 minute. A final extension step also took place at 72°C for 10 minutes. The expected size of the amplified gene product is 309 bp.

Following the polymerase chain reaction, detection of the *mcr-1* amplicon was done using gel electrophoresis. The amplified gene product was inoculated in 1% agarose gel, stained with ethidium bromide, and subjected to electrophoresis for 35 minutes at 100V.

Gene and Primers	**Denaturation**	**Annealing**	**Extension**	**Size**
mcr-1 gene CLR5-F (5′-CGGTCAGTCCGTTTGTTC-3′) and CLR5-R (5′-CTTGGTCGGTCTGTA GGG-3’)	95°C for 1min	56°C for 45 sec	72°C for 1 min	309 bp

Table 1. PCR conditions for the tested *mcr-1* gene

E. Antibiotic Resistance Using the Disc Diffusion Assay

Disc Diffusion Sensitivity method was used to assess antibiotic resistance phenotypes of the *mcr-1 positive E. coli* isolated at the last time point from each experiment. Using Muller Hinton broth, the optical density of the samples was adjusted using a 0.5 McFarland standard. Using sterile cotton swabs, the samples were spread on Muller Hinton agar plates. Nineteen antibiotics were used including penicillin (PEN), ampicillin (AMP), amoxicillin + clavulanic acid (AMC), cefepime (FEP), cefotaxime (CTX), cephalexin (LEX), cefixime (CFM), doripenem (DOR), meropenem (MEM), imipenem (IPM), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), tetracycline (TET), ciprofloxacin (CIP), norfloxacin (NOR), trimethoprim-sulfamethoxazole (SXT), chloramphenicol (CHL), and erythromycin. Four antibiotic discs were added to each plate. The plates are then incubated at 37°C for 18-24 hours. Erythromycin was used as a control due to the natural intrinsic resistance of *E. coli* to this antibiotic. Antibiotic resistance was determined by measuring the diameter of zone of inhibition around each antibiotic disk and compared to Clinical and Laboratory Standards Institute [49, 50].

F. Assessment of Reduction or Increase in Bacterial Load

In order to assess the survival potential of the inoculated bacterial strains in the sample studies, comparing the initial and the final bacterial load (CFU/g) was carried out. This allowed to infer the reduction or increases of microorganisms inoculated in the produce samples, which in turn might indicate the bacterial survival potential as well as the product stability in microbiological aspect. Furthermore, the data was also analyzed to deduce which bacterial strains survived better under similar conditions (produce sample inoculated in and storing temperature).

G. Statistical Analysis

To Evaluate the survival of the test isolates on produce samples at room temperature and 4ºC, ANOVA (analysis of variance) and Bonferroni’s range test was used in SPSS version 22. Produce, time after inoculation, and storing temperature were the categorical variables and the CFU/g of each isolate was the dependent variable. Difference of CFU/g was considered significant when P-value<0.05. Same method of analysis was conducted to evaluate which isolate survived better under similar inoculation conditions (produce and storing temperature).

CHAPTER III

RESULTS

A. Survival of mcr-1-Positive Bacteria in the Produce Samples Studied

1. Survival of mcr-1-Positive Bacterial Strains on Lettuce at Room temperature

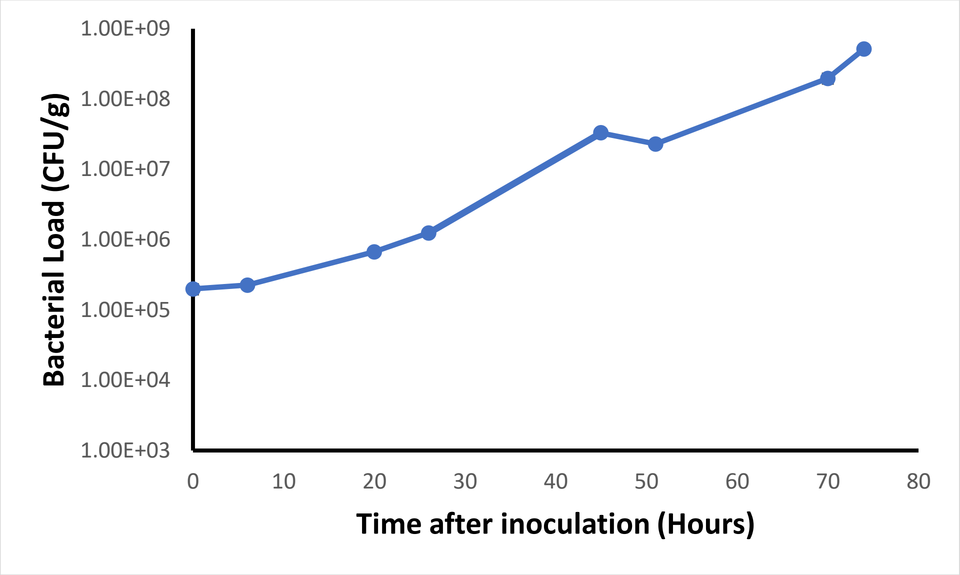

Figure 3. Survival of *mcr-1*-positive *E.coli* isolate of sewage source labeled (WB3) on lettuce at room temperature

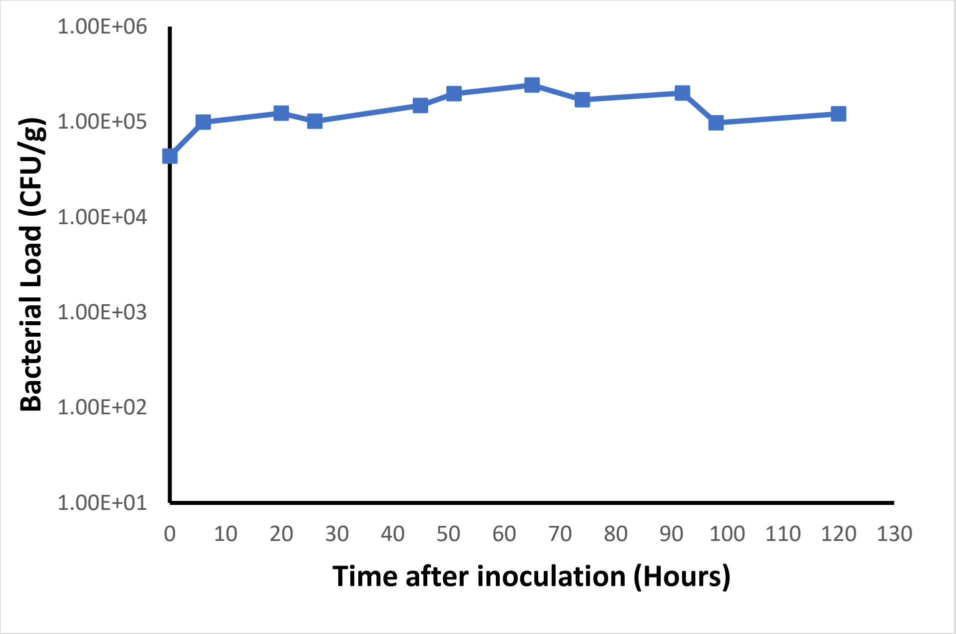

Figure 4. Survival of *mcr-1*-positive *E.coli* isolate of sewage source labeled (WB12 (1)) on lettuce at room temperature

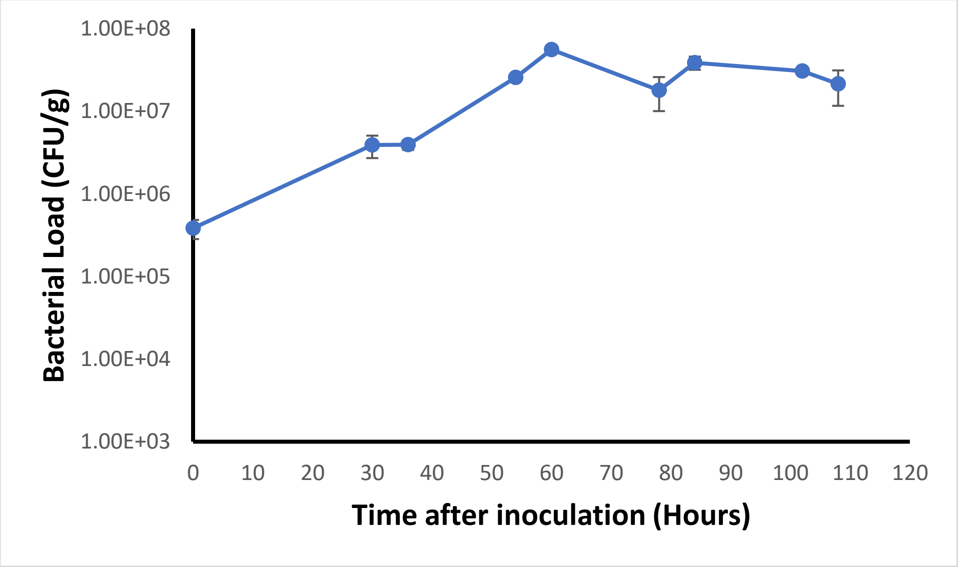

Figure 5. Survival of *mcr-1*-positive *E.coli* isolate of poultry source labeled (65) on lettuce at room temperature

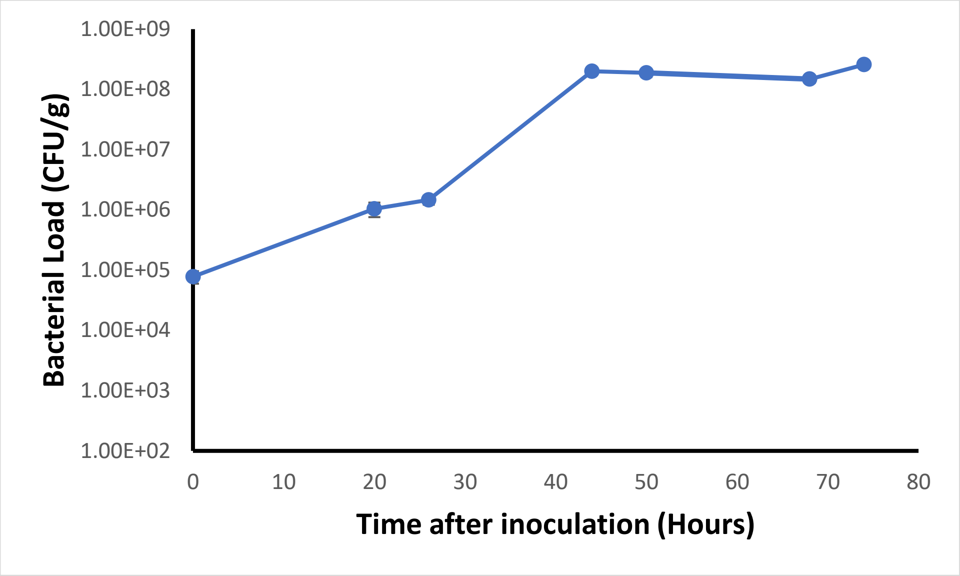

Figure 6. Survival of *mcr-1*-positive *E.coli* isolate of poultry source labeled (58) on lettuce at room temperature

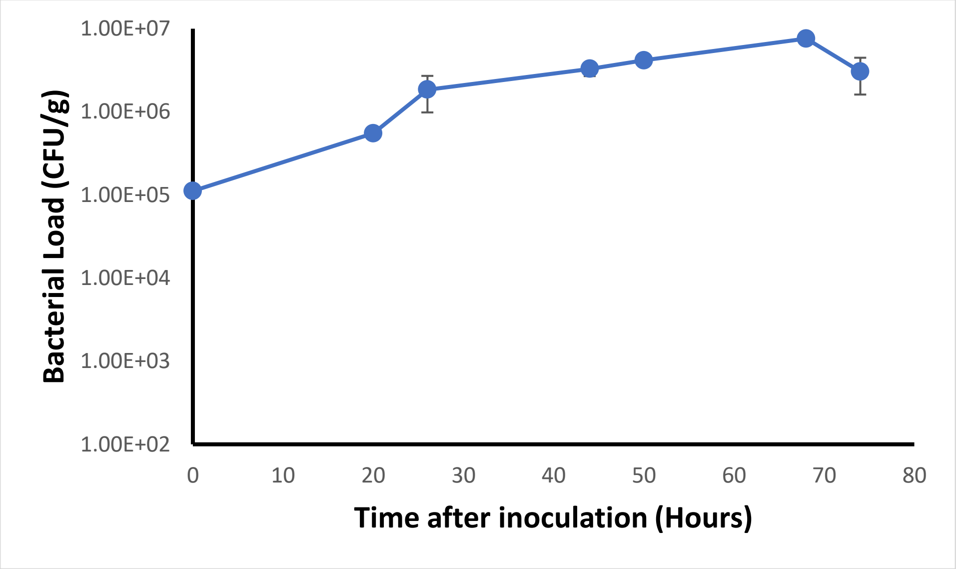

Figure 7. Survival of *mcr-1*-positive *E.coli* isolate of pigeon fecal source labeled (PN41) on lettuce at room temperature

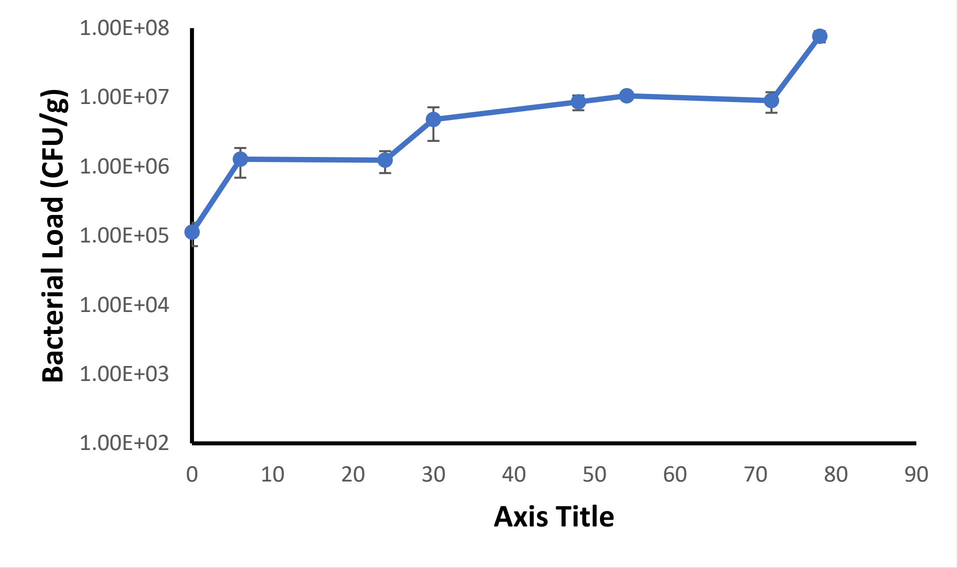

Figure 8. Survival of *mcr-1*-positive *E.coli* isolate of pigeon fecal source labeled (PN43) on lettuce at room temperature

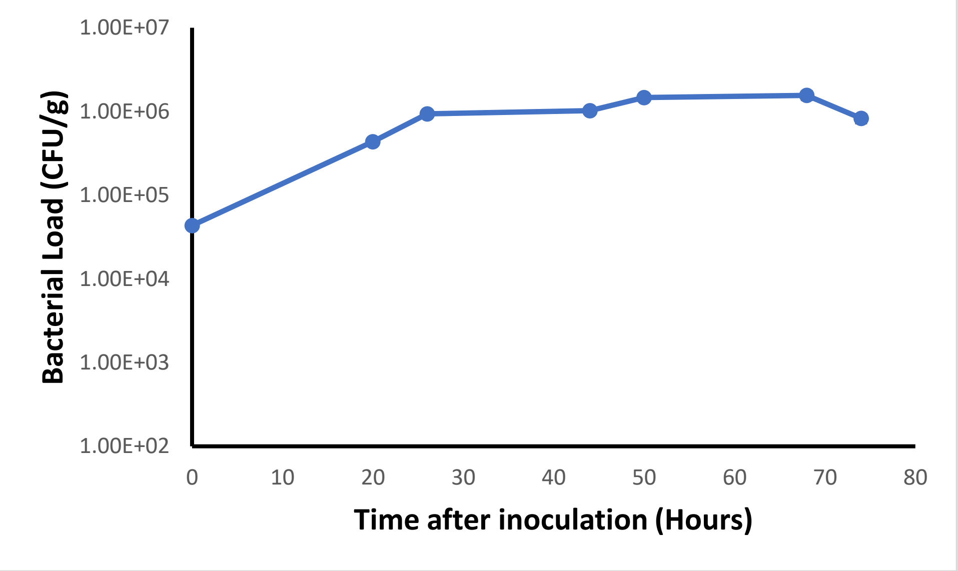

Figure 9. Survival of *mcr-1*-positive *E.coli* isolate of river water source labeled (Waz3 (1)) on lettuce at room temperature

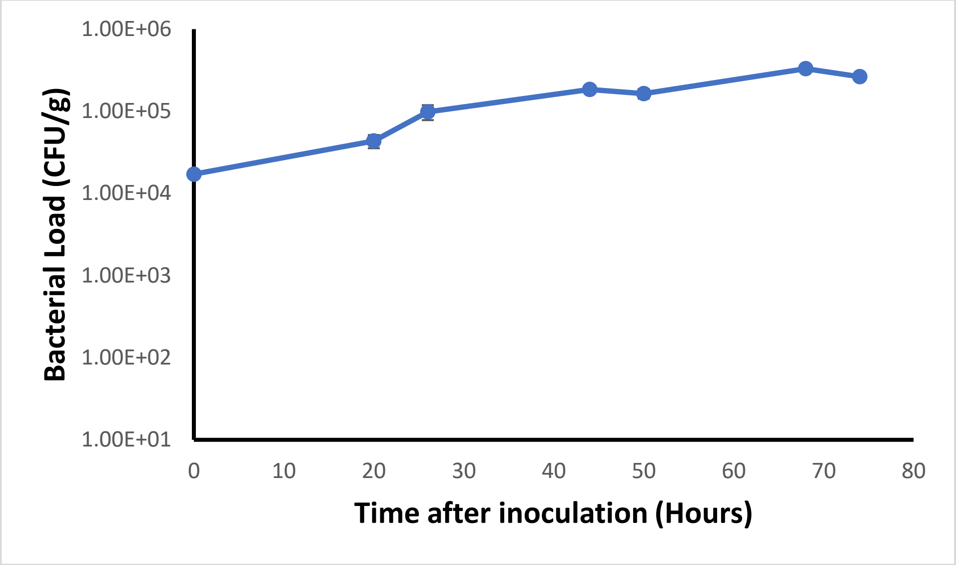

Figure 10. Survival of *mcr-1*-positive *E.coli* isolate of river water source labeled (Waz1 (1)) on lettuce at room temperature

Isolate	**WB3**	**WB12(1)**	**65**	**58**	**PN41**	**PN43**	**Waz3(1)**	**Waz1(1)**
Initial bacterial load (log CFU/g)	**5.30**	**4.64**	**5.58**	**4.89**	**5.05**	**5.03**	**4.64**	**4.23**
Final bacterial load (log CFU/g)	**8.71**	**5.09**	**7.31**	**8.41**	**6.46**	**7.88**	**5.91**	**5.42**

Table 2. Survival of *mcr-1*-harbouring *E.coli* on lettuce at room temperature

Our results portrayed the survival pattern of different *mcr-1*-positive *E.coli* strains in lettuce and spinach samples up to 70-120 hours at room temperature and up to 7-10 days at 4°C. The initial bacterial loads ranged from 10^4-10^5 CFU/g. For the first isolate of sewage source labeled (WB3) inoculated in lettuce at room temperature, the bacterial load increased throughout the first 45 hours after inoculation followed by a slight decrease then another increase (>8 log) towards the last time point. A significant increase in CFU/g was detected between time 0 and time 70 hours ($P<0.05$) and between time 0 and time 74 hours ($P<0.05$). (Fig.3). The second isolate of sewage source labeled (WB12) (1) inoculated in lettuce at room temperature showed a slight increase in bacterial load throughout the first 92 hours after inoculation followed by a slight decrease in bacterial load towards the last time point. (Fig.4) The difference in the mean CFU/g at time 0 and the mean CFU/g at t=120 hours was significant ($P<0.05$). The bacterial load of the isolate of poultry source labeled (65) inoculated in lettuce at room temperature exhibited an increase (>7 log) 60 hours after inoculation followed by a slight decrease at 78 hours then an increase towards the last time point. (Fig.5). The difference of the mean CFU/g at time 0 and the mean CFU/g at t=102 hours was not significant ($P>0.05$); however, the mean CFU/g at time 0 and the mean CFU/g at the last time point t=108 hours was significant ($p<0.05$). The isolate of poultry source labeled (58) exhibited an increase in bacterial load throughout the time of the experiment (10^4-10^8 CFU/g) upon inoculation in lettuce at room temperature (Fig.6). The difference in the mean CFU/g at t=0 the last time point (t=74 hours) was significant ($P<0.05$). The isolate of pigeon fecal source labeled (PN41) inoculated in lettuce at room temperature also exhibited an increase in bacterial load throughout 68 hours after inoculation before a slight decrease at the last time point (t=74 hours) as indicated in (Fig.7). The increase in CFU/g was significant at t=68 hours compared to that at t=0 ($P<0.05$). Similarly, the second isolate of pigeon fecal source labeled (PN43) inoculated in the same conditions exhibited a significant increase in bacterial load at t=78 hours compared to the initial bacterial load at time 0 ($P<0.05$) (Fig.8). Figures 9 and 10 represent the bacterial loads for both *E.coli* isolated from river water source labeled (Waz3 (1)) and (Waz1 (1)) respectively both inoculated in lettuce samples and stored at room temperature. The isolate (Waz3 (1)) exhibited a significant increase in CFU/g when comparing bacterial load at t=0 and t=68 hours ($P<0.05$). The isolate labeled (Waz1 (1)) also inoculated in lettuce at room temperature exhibited a significant increase in CFU/g when comparing bacterial loads at t=0 and the last time point t=74 hours.

Upon comparing the bacterial loads at the last time point of the above isolates inoculated in lettuce and stored at room temperature, we deduce that the isolate of sewage source labeled (WB3) survived better under the aforementioned conditions (> 8 log CFU/g). The final bacterial load of isolate (WB3) was significantly greater than the final bacterial loads of all other isolates when inoculated in lettuce at room temperature. (P<0.05).

2. Survival of mcr-1-Positive Bacterial Strains on Lettuce at 4°C

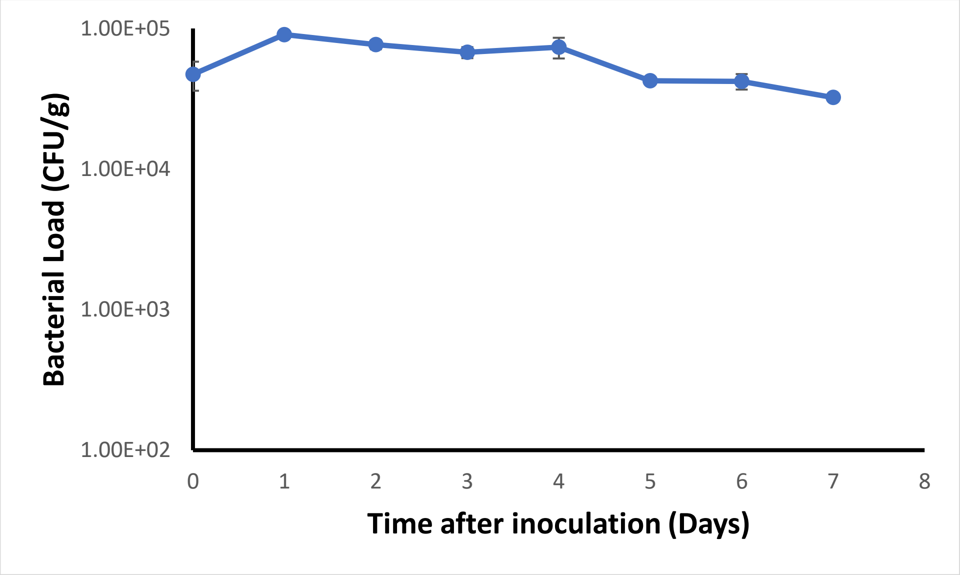

Figure 11. Survival of *mcr-1*-positive *E.coli* isolate of sewage source labeled (WB3) on lettuce at 4°C

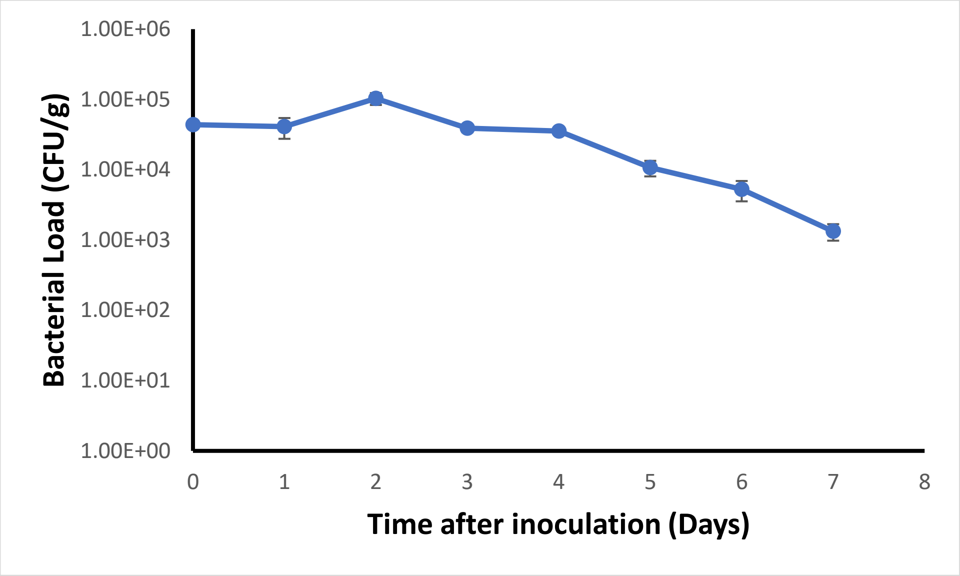

Figure 12. Survival of *mcr-1*-positive *E.coli* isolate of sewage source labeled (WB12 (1)) on lettuce at 4°C

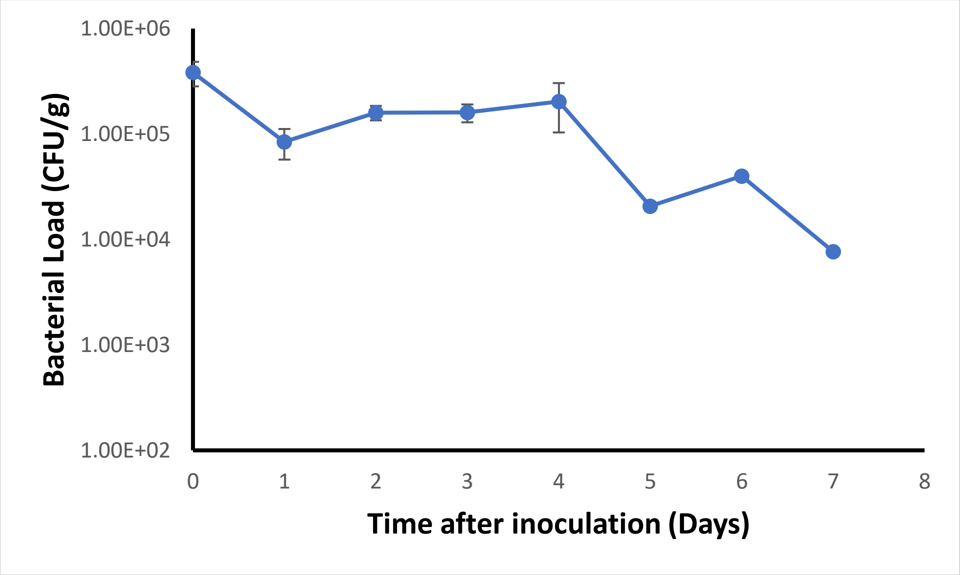

Figure 13. Survival of *mcr-1*-positive *E.coli* isolate of poultry source labeled (65) on lettuce at 4°C

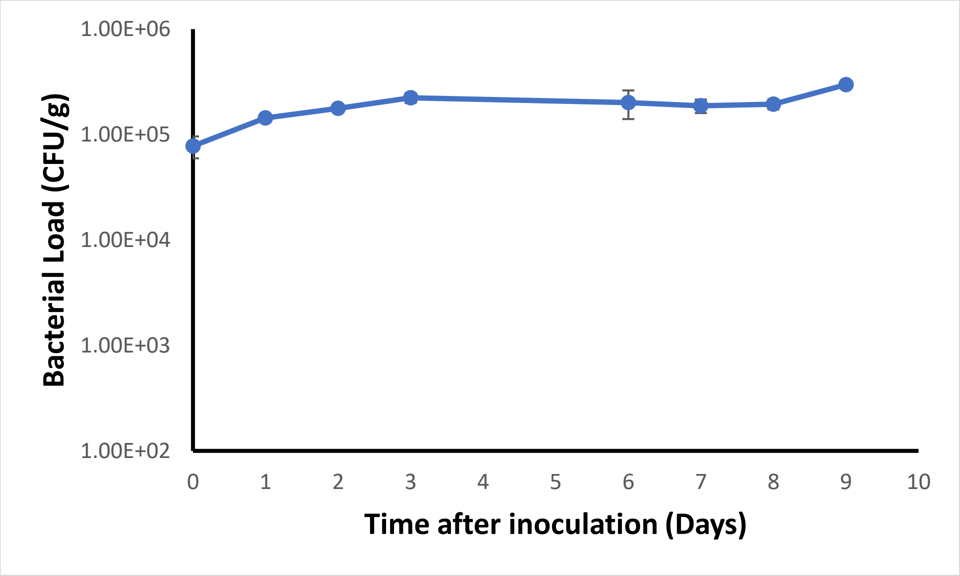

Figure 14. Survival of *mcr-1*-positive *E.coli* isolate of poultry source labeled (58) on lettuce at 4°C

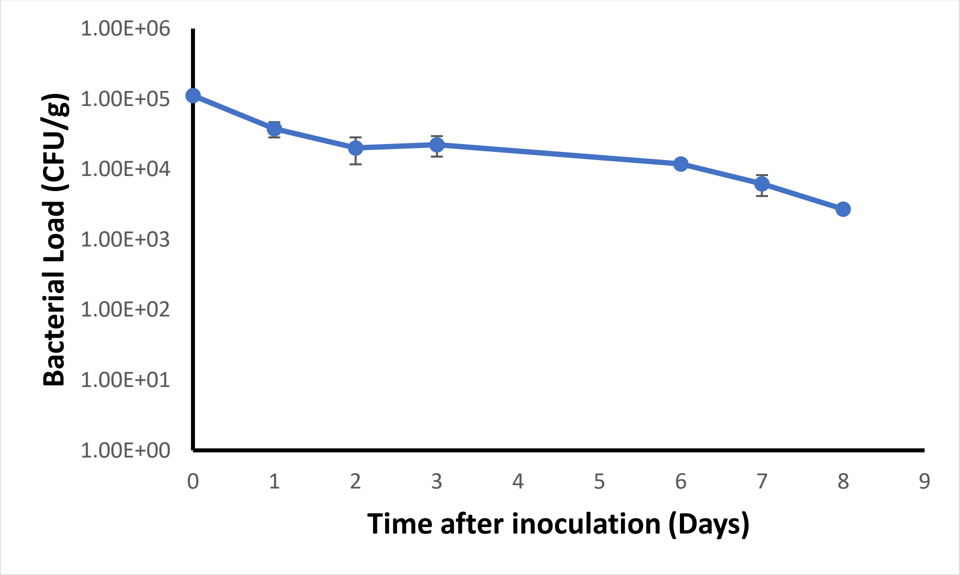

Figure 15. Survival of *mcr-1*-positive *E.coli* isolate of pigeon fecal source labeled (PN41) on lettuce at 4°C

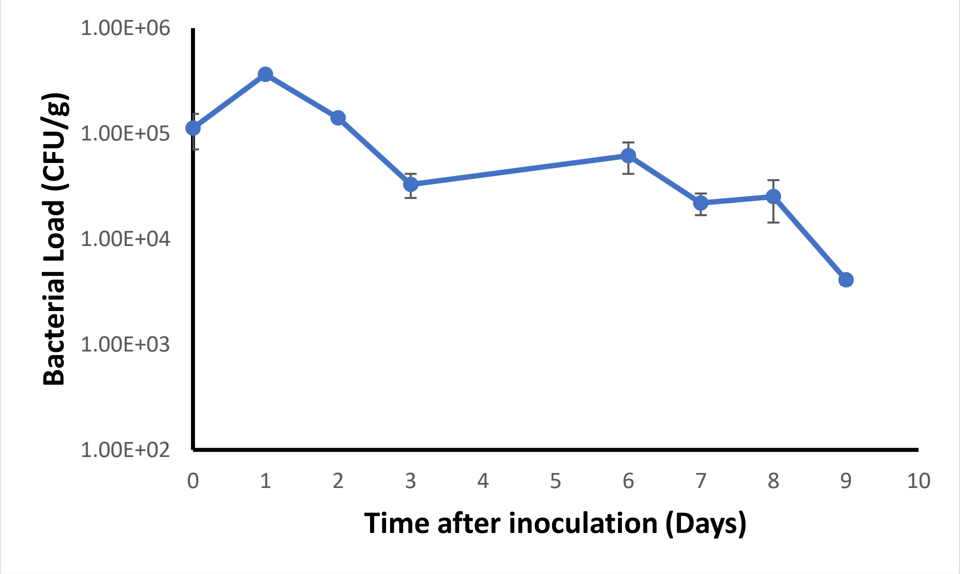

Figure 16. Survival of *mcr-1*-positive *E.coli* isolate of pigeon fecal source labeled (PN43) on lettuce at 4°C

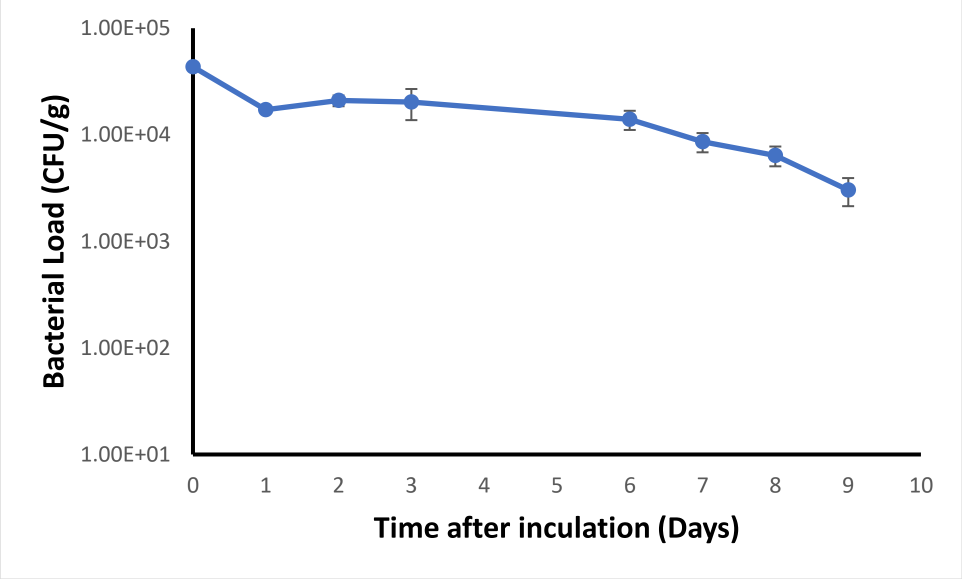

Figure 17. Survival of *mcr-1*-positive *E.coli* isolate of river water source labeled (Waz3 (1)) on lettuce at 4°C

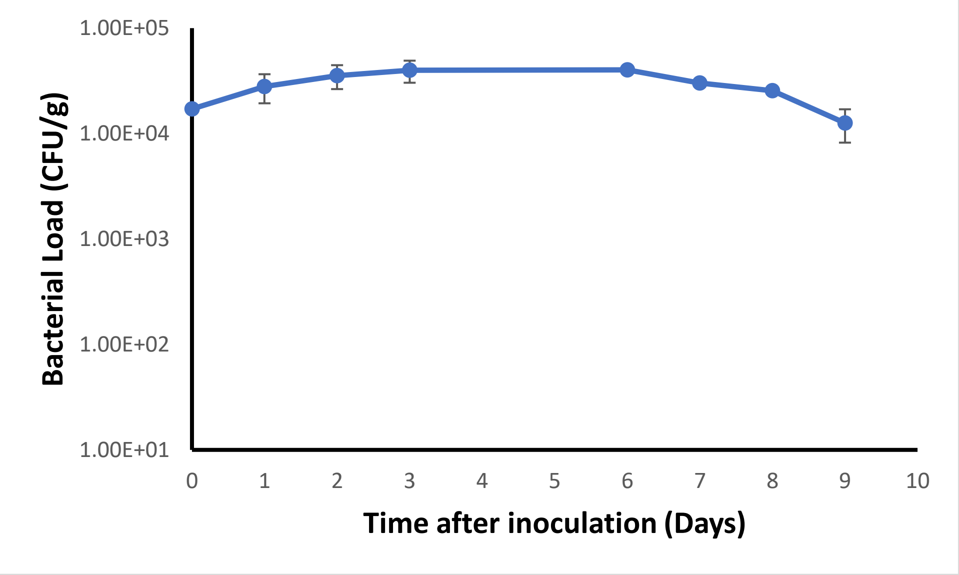

Figure 18. Survival of *mcr-1*-positive *E.coli* isolate of river water source labeled (Waz1 (1)) on lettuce at 4°C

Isolate	**WB3**	**WB12(1)**	**65**	**58**	**PN41**	**PN43**	**Waz3(1)**	**Waz1(1)**
Initial bacterial load (log CFU/g)	**4.67**	**4.64**	**5.58**	**4.89**	**5.05**	**5.03**	**4.64**	**4.23**
Final bacterial load (log CFU/g)	**4.51**	**3.12**	**3.88**	**5.47**	**3.43**	**3.61**	**3.47**	**4.09**

Table 3. Survival of *mcr-1*-harbouring *E.coli* on lettuce at 4°C

Furthermore, the data obtained from assessing the survival of the different bacterial strains inoculated in lettuce at 4°C revealed the survival pattern of these isolates through comparing the initial and final bacterial loads. The first isolate of sewage source labeled (WB3) exhibited a significant increase in bacterial load at t=day1 after inoculation compared to the initial bacterial load ($P<0.05$) followed by nonsignificant decrease in bacterial load throughout the experiment duration when comparing CFU/g values at t=0 and at all other time points including the last time point (day7). ($P>0.05$) (Fig.11). Similarly, the second isolate of sewage source labeled (WB12 (1)) exhibited a significant increase in bacterial load at t= day2 after inoculation compared to the initial bacterial load ($P<0.05$). The final bacterial load recorded at day7 was significantly lower than the initial bacterial load ($P<0.05$) (Fig.12). The poultry isolate labeled (65) inoculated in the same conditions with an initial load of around 10^5 CFU/g exhibited a final bacterial load significantly lower than the initial bacterial load ($P<0.05$) (Fig. 13). Moreover, the second poultry isolate labeled (58), inoculated in similar conditions, exhibited a significantly higher final bacterial load at the last time point (day9) compared to the initial bacterial load recorded at t=o ($P<0.05$) as demonstrated in (Fig.14). The isolate of pigeon fecal source labeled (PN41) inoculated in lettuce at 4°C exhibited a significant decrease in bacterial load at the last time point of the experiment (t= day8) compared to the initial bacterial load recorded at t=0 (Fig.15).

The second isolate of pigeon fecal matter; however, with an initial bacterial load of ~10^5 CFU/g exhibited increases and decreases of bacterial load throughout the duration of the assessment before reaching a final load of around 10^3 CFU/g that is significantly lower than the initial bacterial load. (Fig.16). The isolate of river water source labeled (Waz3 (1)) inoculated in lettuce at 4°C exhibited a final bacterial load at t=day9 that is significantly lower than the initial bacterial load ($P<0.05$). The second isolate of river water source labeled (Waz1(1)) inoculated in the same conditions exhibited a final bacterial load that is not significantly lower that the initial load ($P>0.05$) (Fig.17, Fig.18 respectively).

When comparing the bacterial load at the last time point of the above isolates inoculated in lettuce and stored at 4°C, we deduce that the isolate of poultry source labeled (58) survived better under the aforementioned conditions (final bacterial load > 5 log CFU/g). The difference of the final bacterial load of the isolate (58) was significantly greater than the final bacterial loads of other isolates all inoculated in lettuce at 4ºC ($P<0.05$)

3. Survival of mcr-1-Positive Bacterial Strains on Spinach at Room Temperature

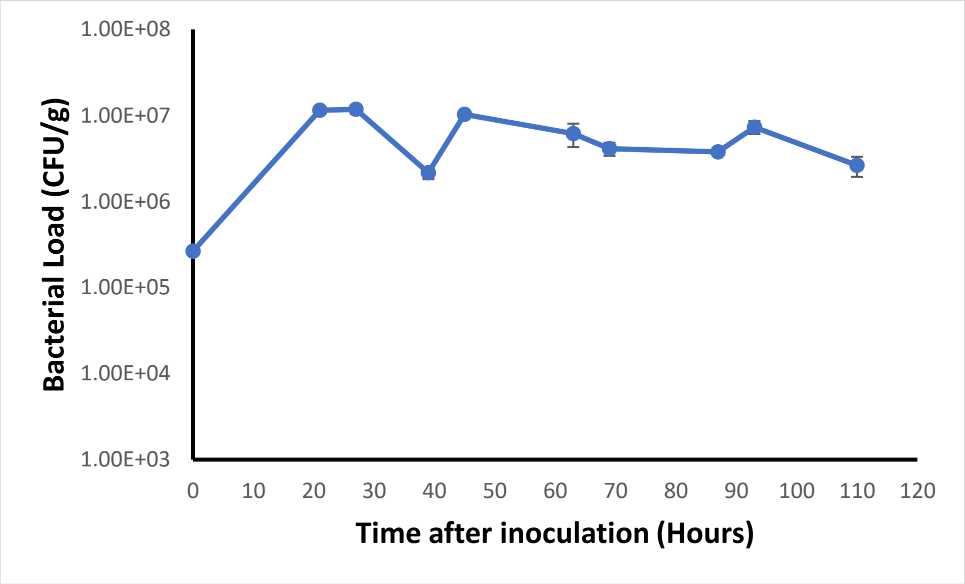

Figure 19. Survival of *mcr-1*-positive *E.coli* isolate of sewage source labeled (WB3) on spinach at room temperature

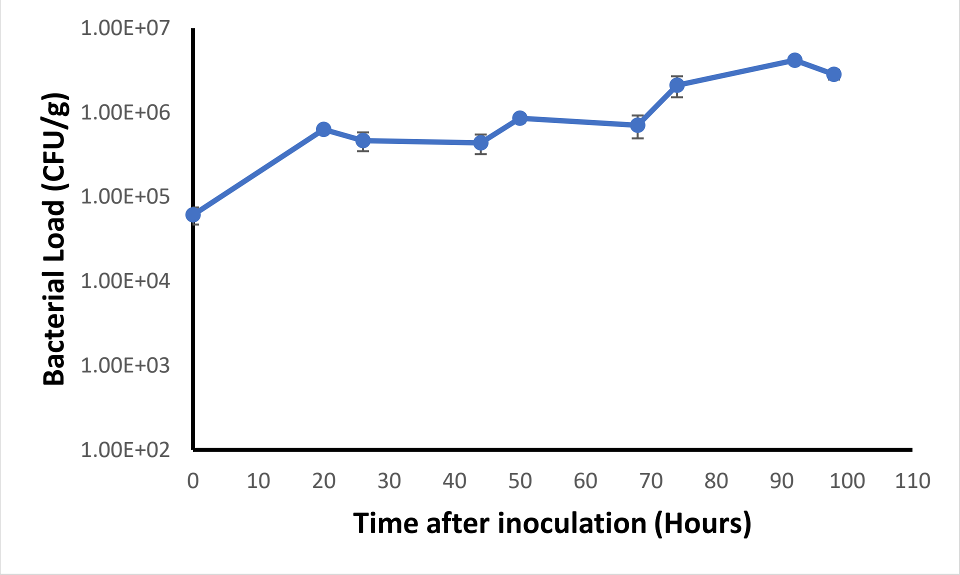

Figure 20. Survival of *mcr-1*-positive *E.coli* isolate of sewage source labeled (WB12 (1)) on spinach at room temperature

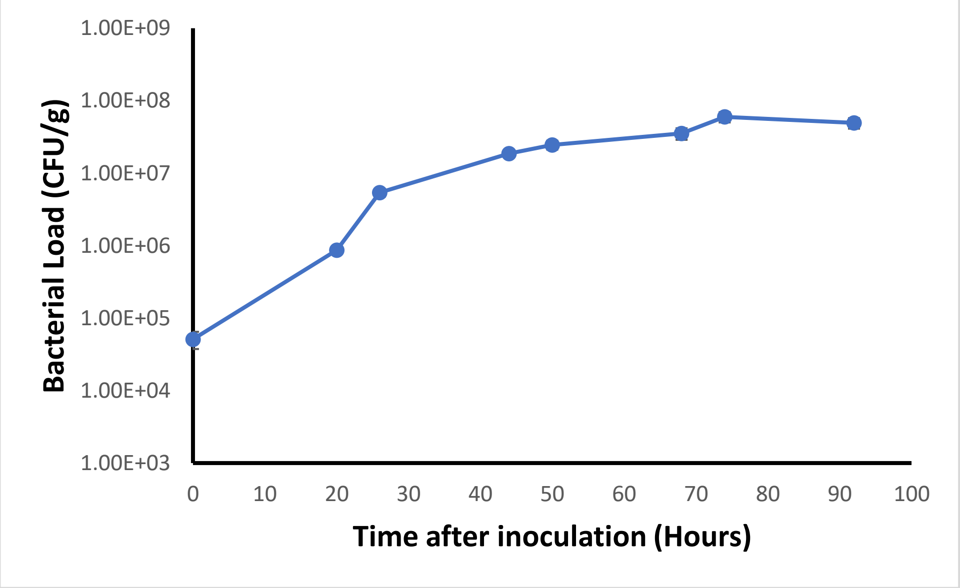

Figure 21. Survival of *mcr-1*-positive *E.coli* isolate of poultry source labeled (65) on spinach at room temperature

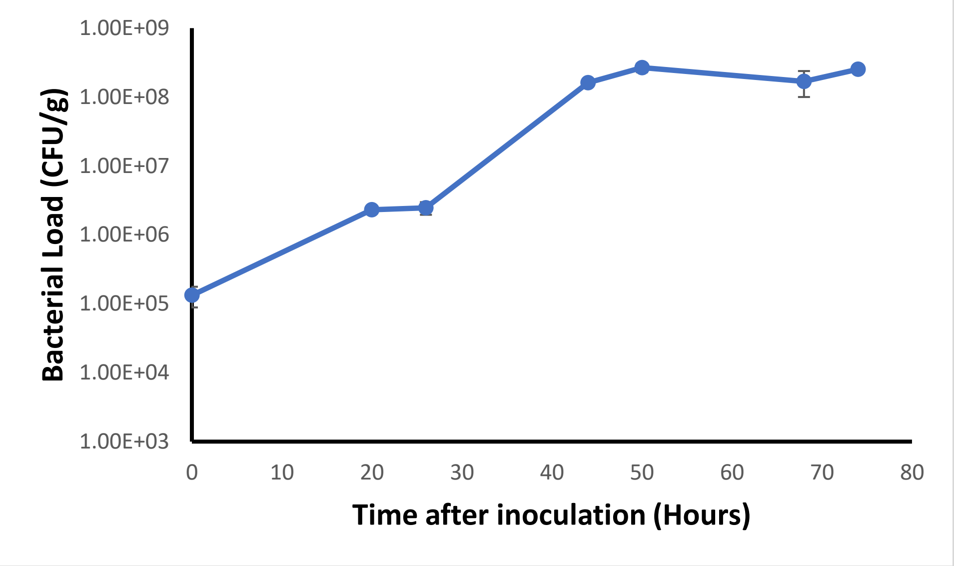

Figure 22. Survival of *mcr-1*-positive *E.coli* isolate of poultry source labeled (58) on spinach at room temperature

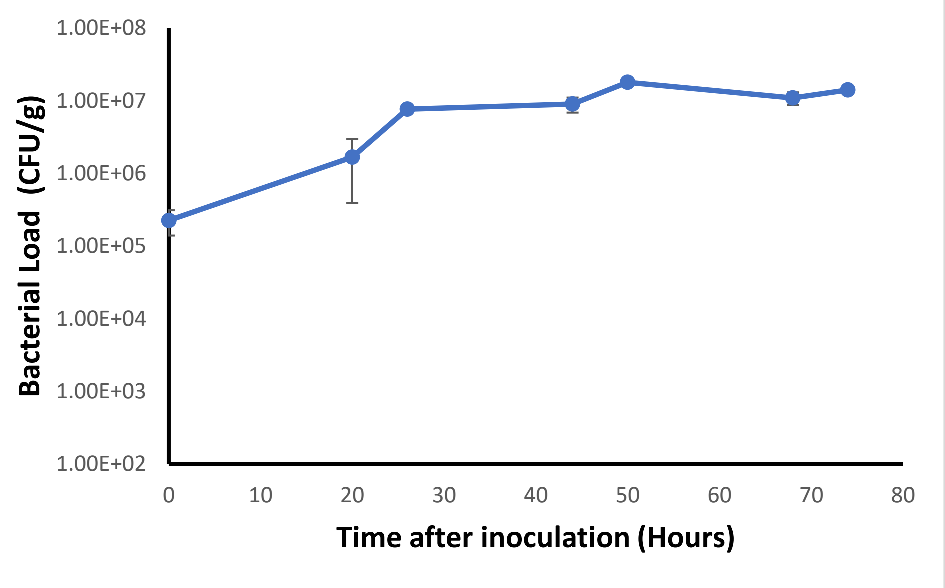

Figure 23. Survival of *mcr-1*-positive *E.coli* isolate of pigeon fecal source labeled (PN41) on spinach at room temperature

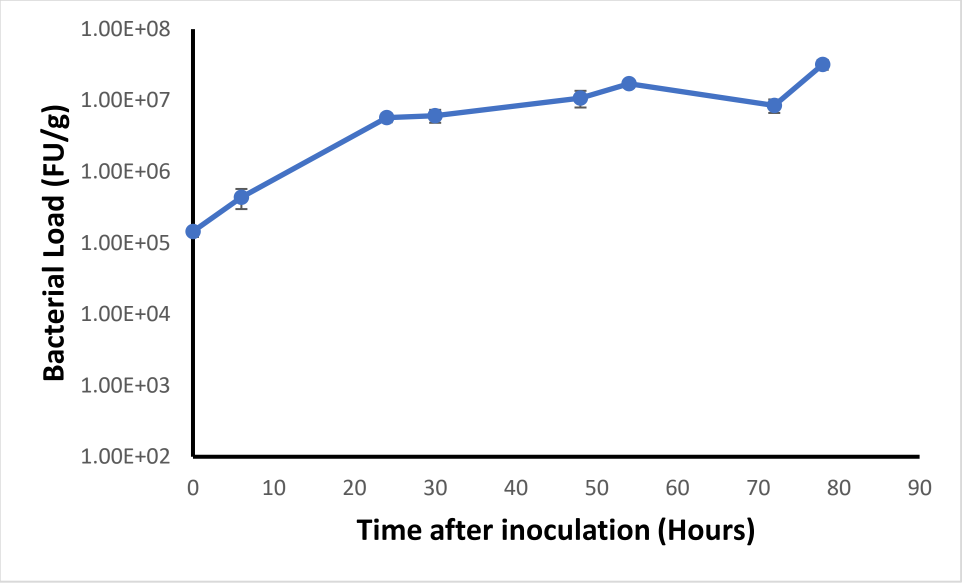

Figure 24. Survival of *mcr-1*-positive *E.coli* isolate of pigeon fecal source labeled (PN43) on spinach at room temperature

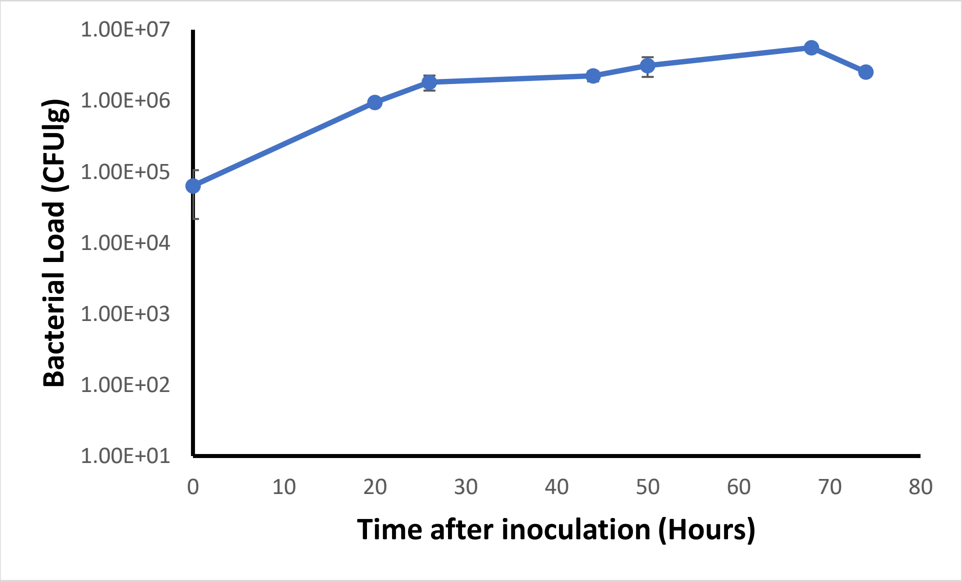

Figure 25. Survival of *mcr-1*-positive *E.coli* isolate of river water source labeled (Waz3 (1)) on spinach at room temperature

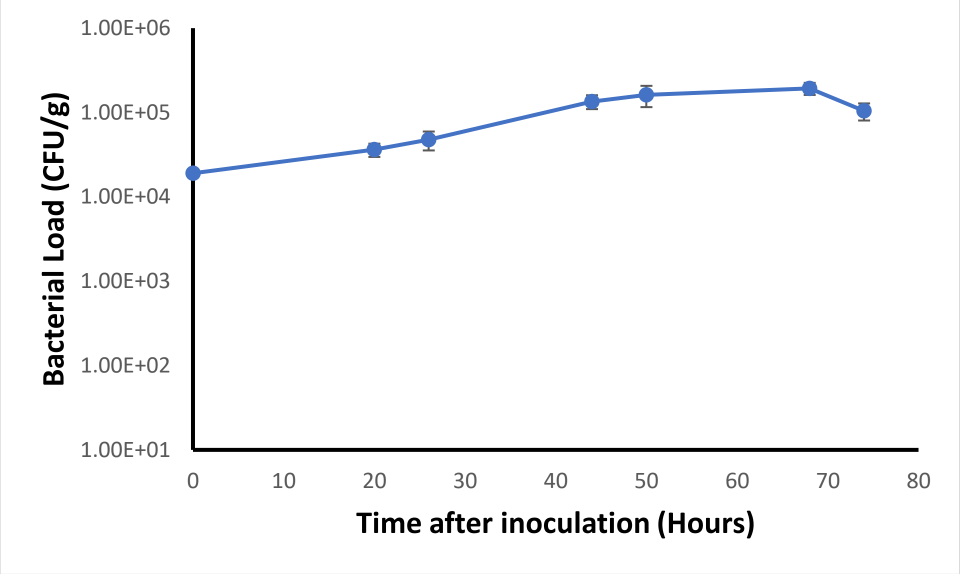

Figure 26. Survival of *mcr-1*-positive *E.coli* isolate of river water source labeled (Waz1 (1)) on spinach at room temperature

Isolate	**WB3**	**WB12(1)**	**65**	**58**	**PN41**	**PN43**	**Waz3(1)**	**Waz1(1)**
Initial bacterial load (log CFU/g)	**5.42**	**4.78**	**4.70**	**5.11**	**5.33**	**5.16**	**4.75**	**4.28**
Final bacterial load (log CFU/g)	**6.41**	**6.45**	**7.69**	**8.40**	**7.15**	**7.50**	**6.40**	**5.01**

Table 4. Survival of *mcr-1*-harbouring *E.coli* in spinach at room temperature

Moreover, the data obtained from assessing the survival of the different bacterial strains inoculated in spinach at room temperature revealed that most of the isolates exhibited a survival potential under the aforementioned inoculation and storing conditions.

The assessment of the survival of the isolate of river source labeled (WB3) on spinach at room temperature showed that the final bacterial load reached 10^7 CFU/g from an initial load of around 10^5 CFU/g at several time points before decreasing to 10^6 CFU/g at the last time point (t= 110 hours) as demonstrated by (Fig.19). The difference between the initial mean bacterial load (t=0) and final mean bacterial load (t=110 hours) is; however, not significant ($P>0.05$). The second isolate of sewage source labeled (WB12 (1)) inoculated in the same conditions exhibited a continues increase in bacterial load (up 10^6 CFU/g) from the initial load of around 10^4 CFU/g before significantly decreasing at the last time point (t=98 hours) compared to the initial mean bacterial load (t=0) ($P<0.05$) (Fig.20). As for the isolate of poultry source labeled (65) also inoculated in spinach at room temperature, the bacterial load had a continuously increasing pattern reaching a final load of more than 10^7 CFU/g from an initial bacterial load of around 10^4 CFU/g. (Fig.21). The difference of the mean CFU/g at the first and last time points was significant ($P<0$). Similarly, the second isolate of poultry source labeled (58) and inoculated in the same conditions, the isolate exhibited a continuous increase in bacterial load reaching a final mean bacterial load of more than 10^8 CFU/g from an initial mean bacterial load of around 10^5 CFU/g.($P<0.05$) (Fig.20). As for the two isolates of pigeon fecal matter (PN41 and PN43) inoculated in the same aforementioned conditions, both isolates exhibited an overall increasing pattern in bacterial load both starting with an initial load of around 10^5 CFU/g and reaching a final load of more than 10^7 CFU/g. (Fig.23, Fig.24) ($P<0.05$). Lastly, the isolate of river source labeled (Waz3(1)) inoculated in spinach at room temperature, exhibited a continuous increase in bacterial load reaching a final mean bacterial load of around10^6 CFU/g from an initial mean bacterial load of around 10^4 CFU/g ($P<0.05$) (Fig.25). As for the second isolate of river water source labeled (Waz1(1)) inoculated in the same conditions, the isolate reached a final mean bacterial load of around10^5 CFU/g from an initial mean bacterial load of around 10^4 CFU/g ($P>0.05$) (Fig.26).

When comparing the mean bacterial loads at the last time point of the above isolates inoculated in spinach and stored at room temperature, we deduce that the isolate of poultry source labeled (58) survived better under the aforementioned conditions (> 8 log CFU/g) with the difference of the mean CFU/g at the last time point of this isolate significantly greater than the mean CFU/g of all other isolates. (P<0.05)

4. Survival of mcr-1-Positive Bacterial Strains on Spinach at 4°C

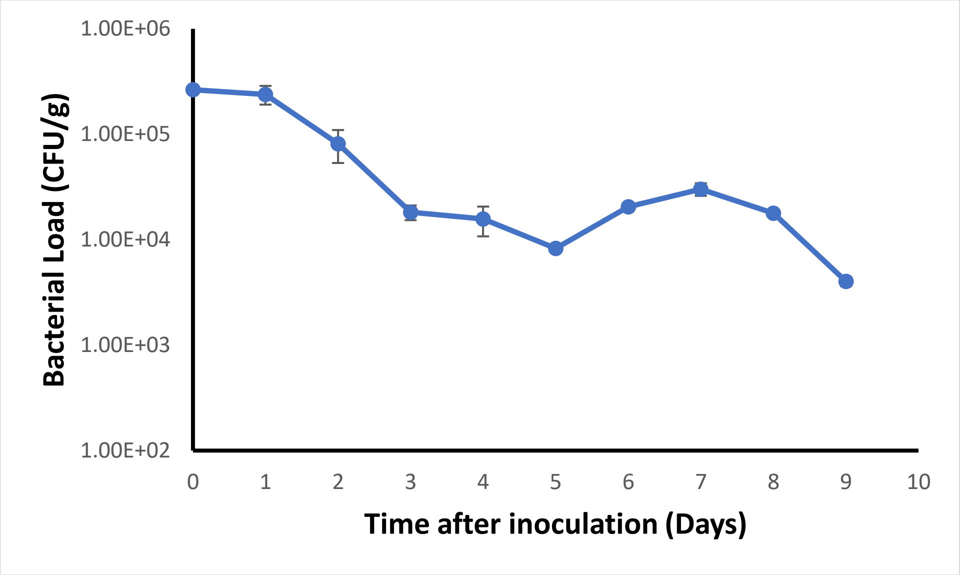

Figure 27. Survival of *mcr-1*-positive *E.coli* isolate of sewage source labeled (WB3) on spinach at 4°C.

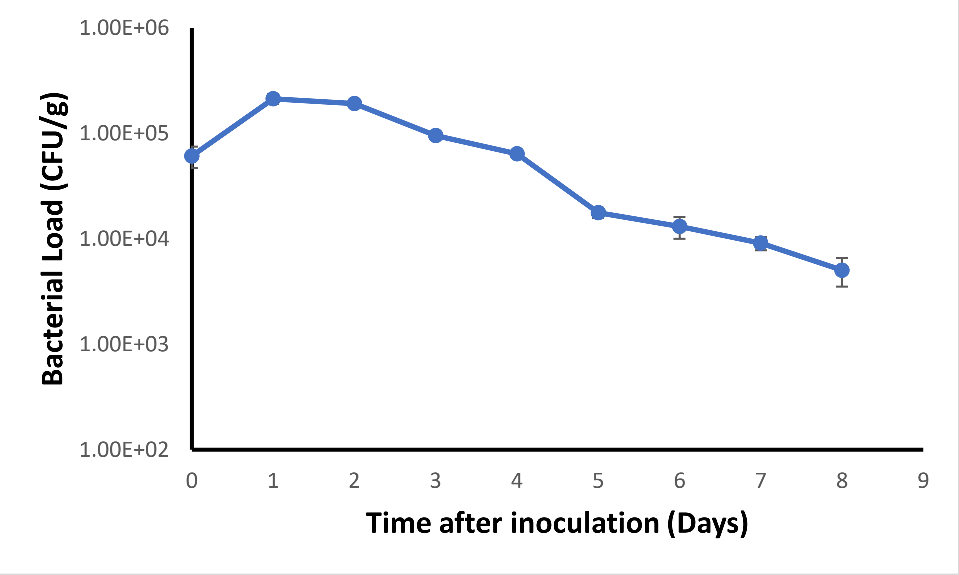

Figure 28. Survival of *mcr-1*-positive *E.coli* isolate of sewage source labeled (WB12(1)) on spinach at 4°C.

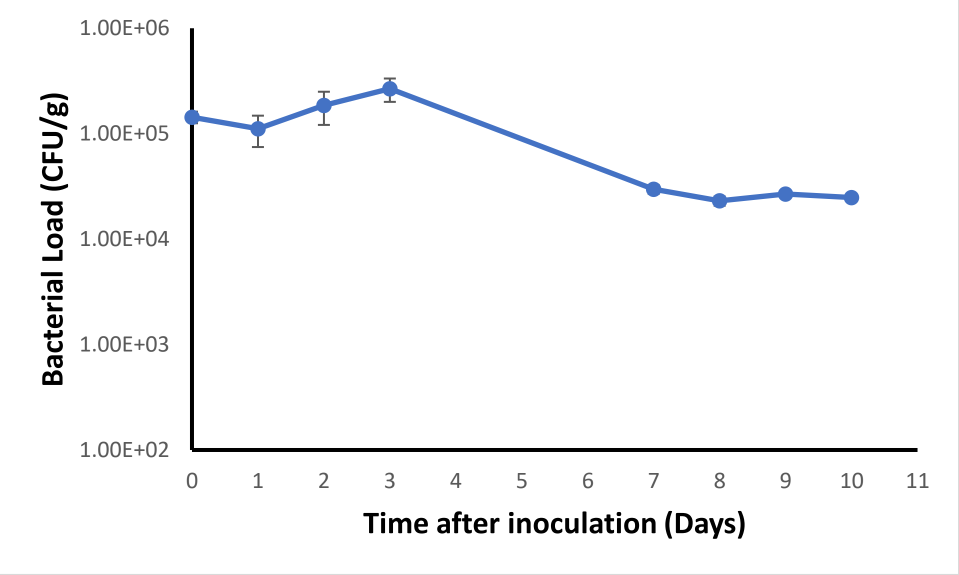

Figure 29. Survival of *mcr-1*-positive *E.coli* isolate of poultry source labeled (65) on spinach at 4°C.

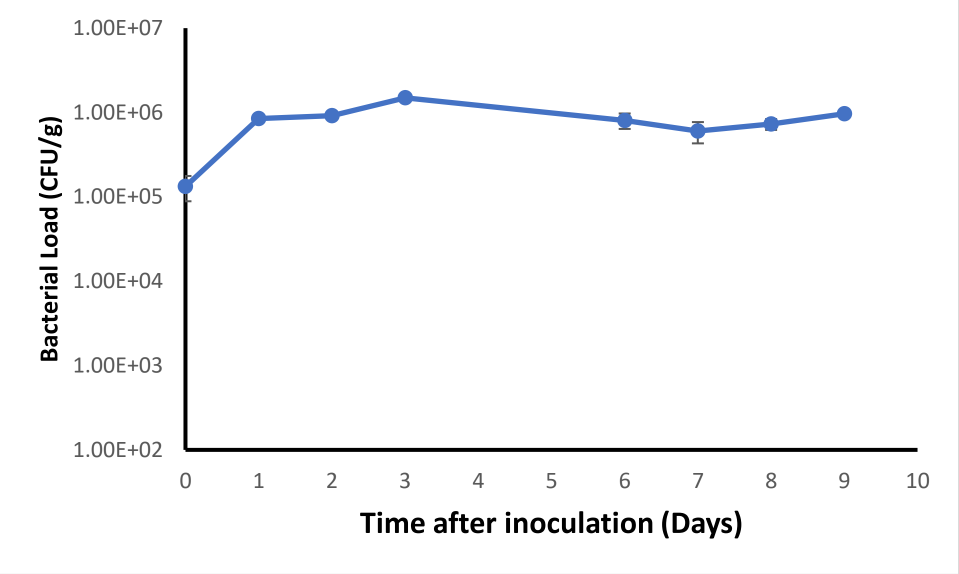

Figure 30. Survival of *mcr-1*-positive *E.coli* isolate of poultry source labeled (58) on spinach at 4°C.

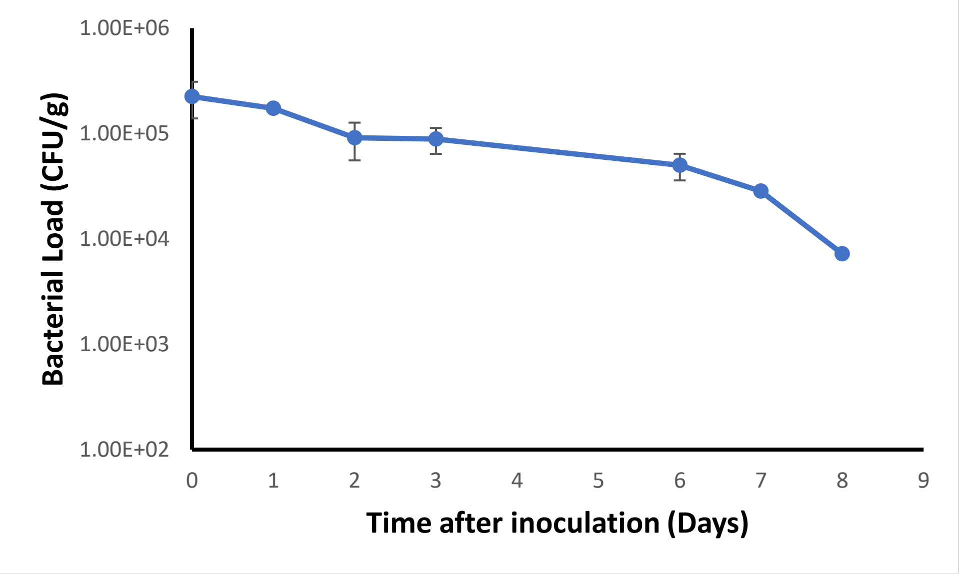

Figure 31. Survival of *mcr-1*-positive *E.coli* isolate of pigeon fecal source labeled (PN41) on spinach at 4°C.

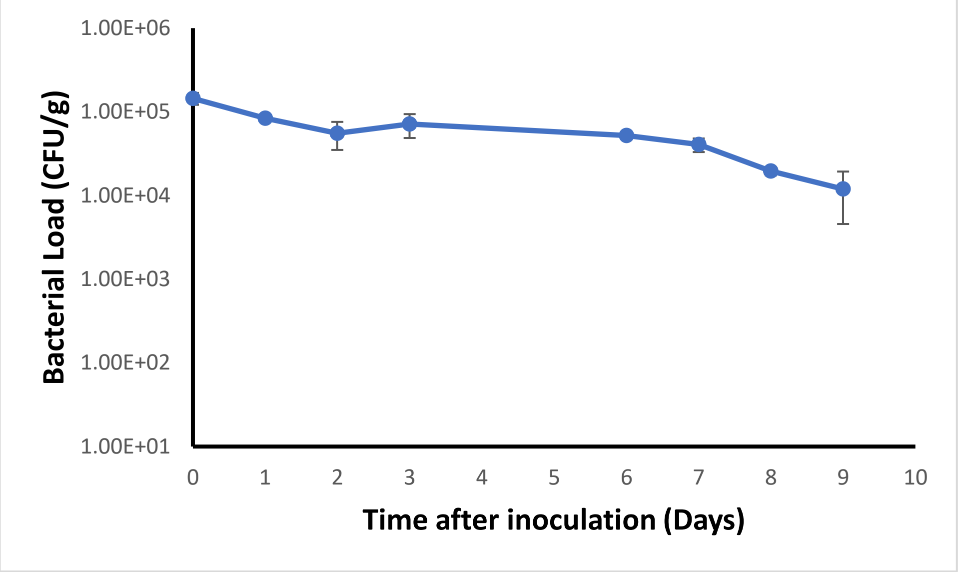

Figure 32. Survival of *mcr-1*-positive *E.coli* isolate of pigeon fecal source labeled (PN43) on spinach at 4°C.

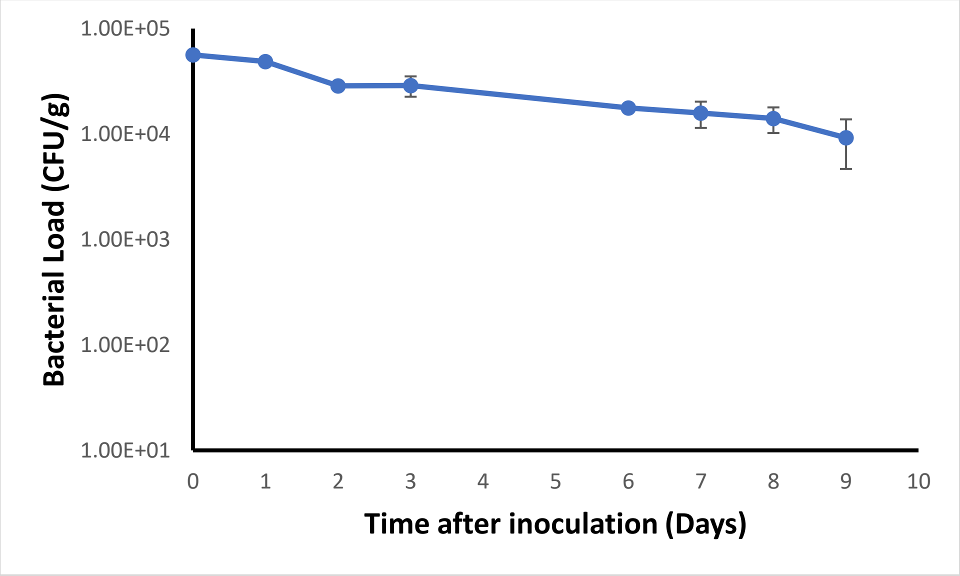

Figure 33. Survival of *mcr-1*-positive *E.coli* isolate of river water source labeled (Waz3(1)) on spinach at 4°C.

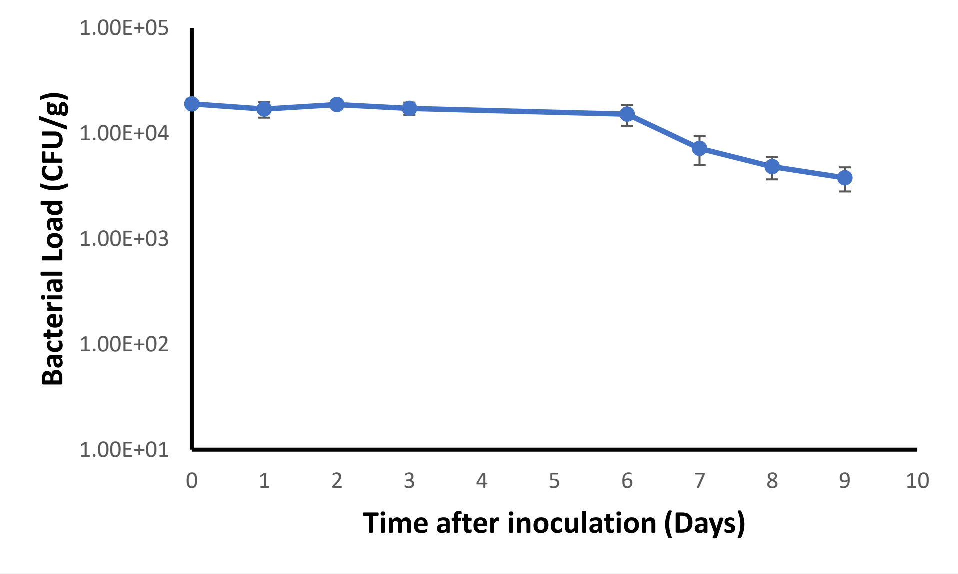

Figure 34. Survival of *mcr-1*-positive *E.coli* isolate of pigeon fecal source labeled (Waz1(1)) on spinach at 4°C.

Isolate	**WB3**	**WB12(1)**	**65**	**58**	**PN41**	**PN43**	**Waz3(1)**	**Waz1(1)**
Initial bacterial load (log CFU/g)	**5.42**	**4.78**	**5.16**	**5.11**	**5.33**	**5.16**	**4.75**	**4.28**
Final bacterial load (log CFU/g)	**3.60**	**3.69**	**4.40**	**5.98**	**3.86**	**4.03**	**3.94**	**3.57**

Table 5. Survival of *mcr-1*-harbouring *E.coli* spinach at 4°C

Lastly, assessing the survival potential of the used *mcr-1*-harbouring *E.coli* inoculated in spinach and stored at 4°C revealed the different patterns in changes of bacterial loads throughout the inoculation time. The first sewage source isolate labeled (WB3) exhibited fluctuations in bacterial load ranging from a continuous decrease the first 5 days after inoculation reaching a lowest mean bacterial load of around 10^3 CFU/g which was significantly lower than the initial mean bacterial load ($P<0.05$). The final mean bacterial load reached around 10^3 CFU/g at the last time point; day 9 which was significantly lower than the initial mean bacterial load ($P<0.05$) (Fig.27). The second isolate of sewage source labeled (WB12(1)) inoculated in similar conditions exhibited a sudden increase in mean bacterial load on day 1 (which was significantly greater than the initial mean bacterial load P<0.05) before exhibiting a continuous decreasing pattern and reaching a final mean bacterial load of around 10^3 CFU/g on the last day; day8 that was significantly lower than the initial mean bacterial load (P<0.05) (Fig.28). As for the isolate of poultry source labeled (65) also inoculated in spinach under refrigeration conditions, the isolate exhibited a slight decrease in bacterial load on day 1 after inoculation before increasing during day 2 and day 3, then decreasing again before almost stabilizing the last four days of inoculation at around 10^4 CFU/g (Fig.29) However, these fluctuations in mean bacterial load were not significant compared to the initial mean bacterial load (P>0.05). Similarly, the isolate of poultry source labeled (58) inoculated in the same conditions exhibited an increase in mean bacterial load the first 3 days of inoculation compared to the initial mean bacterial load (P<0.05). The final bacterial load reached an average CFU/g of around 10^5 that was significantly greater than the initial mean bacterial load (P<0.05) (Fig.30). The two isolates of pigeon fecal source (PN41 and PN43) exhibited a continuous decrease in bacterial load starting with an initial mean bacterial load of around 10^5 CFU/g and reaching a final mean bacterial load of 10^3 CFU/g and 10^4 CFU/g respectively (Fig.31, Fig.32) When comparing the final mean CFU/g of isolate (PN41) to that of the initial mean bacterial load, the difference was significant (P<0.05). Similarly, the final mean bacterial load of isolate (PN43) was significantly lower than the initial mean bacterial load (P<0.05). Lastly, the isolate of river water source labeled (waz3(1)) reached a final mean bacterial load that was significantly lower than the initial bacterial load (P<0.05) (Fig.33). Similarly, the isolate of river water source labeled (Waz1(1)) reached a final mean bacterial load that is significantly lower than the initial mean bacterial load (P<0.05) as demonstrated in (Fig.34).

When comparing the bacterial loads at the last time points of the above isolates inoculated in spinach and stored at 4°C, we deduce that the isolate of poultry source labeled (58) survived better under the aforementioned conditions with a final mean bacterial load of around 6 log CFU/g that was significantly greater than the final bacterial loads of other bacterial isolates ($p<0.05$).

B. The Effect of Produce Samples on the Survival of *mcr-1*-Positive *E.coli*

The final bacterial load was used to compare the survival of the bacterial isolates on lettuce and spinach under the same storing temperature conditions:

1. Room Temperature

The first isolate of sewage source (WB3) reached a final bacterial load of (> 8 log CFU/g) when inoculated in lettuce compared to the final bacterial load of the same isolate inoculated in spinach (> 6 log CFU/g). On the other hand, the second isolate of sewage source (WB12(1)) reached a final bacterial load of (> 6 log CFU/g) when inoculated in spinach compared to a final bacterial load of (5 log CFU/g) when inoculated in lettuce. As for the isolates of poultry source, the isolate labeled (65) reached a final bacterial load of (>7.5 log CFU/g) when inoculated in spinach compared to the final bacterial load of the same isolate inoculated in lettuce (>7 log CFU/g). The second isolate of poultry source (labeled 58); however, reached the same final bacterial load of (>8 log CFU/g) once inoculated in lettuce and in spinach. The isolate of pigeon fecal source labeled (PN43) reached a final bacterial load of ($>10^7$) when inoculated both in lettuce and spinach. The second isolate of pigeon fecal source labeled (PN41); however, reached a final bacterial load (>7 log CFU/g) compared to a final bacterial load of (> 6 log CFU/g) when inoculated in lettuce. Similarly, the isolate of river water source labeled (Waz3 (1)) reached a final bacterial load of (> 6 lo CFU/g) when inoculated in spinach compared to a final bacterial load of (> 5 log CFU/g) when inoculated in lettuce. The second isolate of river water source labeled (Waz1 (1)); however, reached a final bacterial load of (>5 log CFU/g) when inoculated in both spinach and lettuce.

Inoculation produce sample	**Lettuce**	**Spinach**
Final bacterial load (log CFU/g)		
WB3	**8.71**	**6.41**
WB12 (1)	**5.08**	**6.45**
65	**7.31**	**7.69**
58	**8.41**	**8.40**
PN41	**6.46**	**7.15**
PN43	**7.88**	**7.50**
Waz3 (1)	**5.91**	**6.40**
Waz1 (1)	**5.42**	**5.01**

Table 6. Survival of *mcr-1*-harbouring *E.coli* on lettuce and spinach at room temperature

2. Refrigeration Conditions (4°C)

The first isolate of sewage source (WB3) reached a final bacterial load of (> 4 log CFU/g) when inoculated in lettuce compared to the final bacterial load of the same isolate inoculated in spinach (> 3 log CFU/g). On the other hand, the second isolate of sewage source (WB12(1)) reached a final bacterial load of (almost 4 log CFU/g) when inoculated in spinach compared to a final bacterial load of (3 log CFU/g) when inoculated in lettuce. As for the isolates of poultry source, the isolate labeled (65) reached a final bacterial load of (>4 log CFU/g) when inoculated in spinach compared to the final bacterial load of the same isolate inoculated in lettuce (>3 log CFU/g). Similarly, isolate (58) of poultry source reached a final bacterial load of almost (6 log CFU/g) when inoculated in spinach compared to a final bacterial load of (>5 log CFU/g) when inoculated in lettuce. As for the isolates of pigeon fecal source, isolate (PN43) reached a final bacterial load of (>4 log CFU/g) when inoculated in spinach compared to a final bacterial load of (>3 log CFU/g) when inoculated in lettuce. As for the second isolate of pigeon fecal source labeled (PN41), the final bacterial load when the isolate was inoculated in spinach almost reached 4 log CFU/g while it was more than 3 log CFU/g when the isolate was inoculated in lettuce. Similarly, the isolate of river water source labeled (Waz3 (1)) reached a final bacterial load of (almost 4 log CFU/g) when inoculated in spinach compared to a final bacterial load of (> 3 log CFU/g) when inoculated in lettuce. The second isolate of river water source labeled (Waz1 (1)); however, reached a final bacterial load of (>4 log CFU/g) when inoculated in lettuce compared to a final bacterial load of (>3 log CFU/g) when inoculated in spinach.

Inoculation produce sample	**Lettuce**	**Spinach**
Final bacterial load (log CFU/g)		
WB3	**4.51**	**3.60**
WB12 (1)	**3.12**	**3.69**
65	**3.88**	**4.40**
58	**5.47**	**5.98**
PN41	**3.43**	**3.86**
PN43	**3.61**	**4.03**
Waz3 (1)	**3.47**	**3.94**
Waz1 (1)	**4.09**	**3.57**

Table 7. Survival of *mcr-1*-harbouring *E.coli* on lettuce and spinach at 4°C

C. The Effect of Temperature on the Survival of *mcr-1*-Positive *E.coli* in Lettuce and Spinach

As predicted, by comparing the final bacterial loads of the same isolate inoculated in the same produce sample but stored at different temperatures, all isolates exhibited higher final bacterial loads when inoculated at room temperature compared to the final bacterial loads reached at 4ºC (Table.8)

	Final bacterial load (log CFU/g)			
	Inoculated in lettuce		**Inoculated in spinach**	
Isolate	**Room temperature**	**4°C**	**Room temperature**	**4°C**
WB3	**8.71**	**4.51**	**6.41**	**3.60**
WB12 (1)	**5.08**	**3.11**	**6.45**	**3.69**
65	**7.31**	**3.88**	**7.69**	**4.40**
58	**8.41**	**5.47**	**8.40**	**5.98**
PN41	**6.46**	**3.43**	**7.15**	**3.86**
PN43	**7.88**	**3.61**	**7.50**	**4.03**
Waz3 (1)	**5.91**	**3.47**	**6.40**	**3.94**
Waz1 (1)	**5.42**	**4.09**	**5.01**	**3.57**

Table 8. Survival of *mcr-1*-harbouring *E.coli* on lettuce and spinach at room temperature and at 4°C

D. PCR Analysis to Detect the Persistence of *mcr-1* on Produce

The survived isolates from the last time points from lettuce and spinach at room temperature and at 4°C all showed positive *mcr-1* bands on PCR analysis. Four isolates from each time point were tested for *mcr-1* gene. All tested isolates from each last time point (n=4) retained the *mcr-1* gene.

E. The Impact of *mcr-1* Persistence on AMR Profiles

Minor changes in AMR profiles were recorded when comparing the original isolates to the survived isolates. The survived isolates from sewage source labeled WB3 had identical AMR profiles compared to the original isolate. Both isolates showed the

AMR profile below; PEN-AMP-AMC-LEX-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL.

Similarly, the survived isolates from river water source labeled Waz 3 (1) had identical AMR profiles to that of the original isolate. Both isolates showed the AMR profile below; PEN-AMP-AMC-FEP-CTX-LEX-CFM-GEN-KAN-STR-TET-CIP-NOR-SXT-CHL.

On the other hand, the survived isolates of sewage source labeled WB 12 (1); had an AMR resistance profile PEN-AMP-AMC-FEP-CTX-LEX-CFM -TET-CIP-NOR-SXT-CHL and were not resistant to doripenem (DOR), kanamycin (KAN), and streptomycin (STR) while the original isolate had an AMR resistance profile PEN-AMP-AMC-CTX-LEX-CFM-DOR-KAN-STR-TET-SXT-CHL and an intermediate profile FEP-GEN-CIP-NOR. The survived isolates did not show an intermediate resistance profile to cefepime (FEP), gentamicin (GEN), ciprofloxacin (CIP), and norfloxacin (NOR) while the original isolate had an intermediate profile for these antibiotics. The survived isolates were also resistant to cefepime (FEP), ciprofloxacin (CIP), and norfloxacin (NOR) which was not the case for the original isolate.

As for the isolates of poultry source, minor changes on AMR profiles were also recorded when comparing the survived isolates to the original ones. The survived isolates had an AMR profile PEN-AMP-AMC-LEX-GEN-KAN-STR-TET-CHL. The original isolate had an AMR profile PEN-AMP-AMC-CFM-GEN-KAN-STR-TET-CHL. The difference in the AMR profile included resistance to cefixime (CFM) and cephalexin (LEX) in the original and survived isolate respectively, both labeled (65). As for the second isolate of poultry source labeled (58), the survived isolates had an AMR resistance profile PEN-AMP-AMC -LEX-CFM-GEN-KAN-STR-TET -SXT-CHL and an intermediate profile CIP while the original isolate had an AMR profile PEN-AMP-AMC-CTX-LEX-CFM-GEN-KAN-STR-TET-SXT-CHL. The survived isolates were not resistant to cefotaxime (CTX) whereases the original isolate was resistant to the antibiotic. On the other hand, the survived isolates had an intermediate resistance profile to ciprofloxacin (CIP) which was not the case for the original isolate.

Moreover, the isolates of pigeon fecal source revealed minor changes of AMR profiles when comparing the survived isolates to the original isolates. The survived isolates labeled (PN41) had an AMR resistance profile PEN-AMP-AMC-LEX-GEN -TET -SXT-CHL and an intermediate profile KAN-STR while the original isolate had an AMR profile PEN-AMP-AMC-LEX-GEN -SXT-CHL. The survived isolates were resistant to tetracycline (TET) and had intermediate resistance profiles to kanamycin (KAN) and streptomycin (STR) which was not the case for the original isolate. The second original isolate of the same source labeled (PN43), with an AMR profile PEN-AMP-AMC-CTX-LEX-CFM-GEN-KAN-STR-TET-SXT-CHL, was resistant to cefotaxime (CTX), cefixime (CFM), and kanamycin (KAN) while the survived isolates with an AMR resistance profile PEN-AMP-AMC-LEX-GEN-STR-TET -SXT-CHL and an intermediate profile KAN, were not resistant to cefotaxime (CTX), cefixime (CFM), and kanamycin (KAN). The survived isolates; however, had an intermediate resistance profile to kanamycin (KAN).

The original isolate of river source labeled (waz1(1)) with an AMR profile PEN-AMP-AMC-FEP-CTX-LEX-CFM-GEN-KAN-TET-CIP-SXT-CHL, was not resistant to streptomycin (STR) and norfloxacin (NOR) whereases the survived isolates had an AMR profile PEN-AMP-AMC-FEP-CTX-LEX-CFM-GEN-KAN-STR-TET-CIP-

NOR-SXT-CHL, were resistant to these antibiotics streptomycin (STR) and norfloxacin (NOR).

CHAPTER IV

DISCUSSION

The increasing production of fresh produce to meet the increasing demand within the shortest periods of time has on the other hand increased the risks of contaminations of these elements with pathogenic microorganisms, compromising the consumers safety. Fresh produce comprises a substantial nutritional value and can help preventing several chronic diseases such as obesity, diabetics, and heart diseases. Consuming raw or slightly cooked vegetables has been recognized as a major path of transmitting human pathogens and an important source for several food-borne infections. Fresh vegetables can be contaminated at either preharvest stages; from soil, irrigation water, fecal matter, manure, dust, and human interaction with the produce, or at postharvest stages; from feces, harvesting tools, handling, domestic and wild animals, insects, processing, and rinsing water. [51]

A. AMR and MDR bacteria

Antimicrobial resistance has led to the ineffectiveness of clinical antimicrobial therapy and brought up a major challenge to public health. Humans can acquire antimicrobial resistance from drugs through different sources including food chain or the environment. Although antimicrobials have been regularly supplied in agriculture and food producing animals to increases productivity, the overuse of these drugs has significantly contributed to the emergence of multidrug resistance bacteria. [52]

Colistin has been extensively used in agriculture and food producing animals. It is considered a last resort antibiotic against multidrug-resistant Gram-negative bacteria such as carbapenemase-producer *Enterobacterales*, and *Pseudomonas aeruginosa*. Mobile colistin gene *mcr-1* has been reported in isolates from both human and animal sources. Thus, research has been conducted to actively reduce colistin resistance. [53]

A study conducted in South Korea in 2018 aimed to evaluate the prevalence of and the antimicrobial susceptibility of *mcr*-harboring *Enterobacteriaceae* in retail vegetables and food producing animals. The study revealed the contamination of tested vegetables with *mcr-1*-harboring *E.coli*. All *mcr-1* positive isolates exhibited multidrug resistance and co-produced β-lactamases. The results demonstrated the emergence of *mcr-1* gene in vegetables emphasizing the urge to continuously controlling and monitoring colistin-resistance *Enterobacteriaceae* to limit their transmission to humans through the food chain. [54]

In this study, the survival on both lettuce and spinach of 8 multidrug-resistant, *mcr-1*-harboring *Escherichia coli* from 4 different sources including sewage water, poultry, pigeon fecal matter, and river water, was evaluated. Information on the survival potential of *mcr-1*-harbouring *E.coli* on vegetables that are consumed raw may help in lowering the risk of food poisoning and thus contribute to food safety and quality. The results showed that all tested isolates survived up to 72-120 hours at room temperature and up to 7-10 days under refrigeration conditions in both types of fresh produce used. In some tested samples, after a period of a gradually declining bacterial load, sudden increase in bacterial load was observed before returning to another decreasing pattern. One possible reason could be that the sample were tested beyond its shelf life, or stored in conditions that promoted spoilage, leading to a period in which bacterial cells recovered resulting in increased bacterial load in product. [55]. Some *E.coli* isolates exhibited high survival potential on both lettuce and spinach. This could be an alarming fact, given that these isolates are multidrug resistant and harbor the mobile colistin gene *mcr-1*, thus contributing to the spread and dissemination of this gene. This could also indicate high levels of support for the growth of organisms, which can be easily transmitted through fecal contamination. Frequent exposure to enteric diseases upon consumption of raw vegetables might be thus explained through the survival of these bacteria. [56]

The decrease in bacterial load could be due to the normal growth inhibitory strategy which results from the turning down of several sets of genes in the stationary phase [57] or due to the limiting substrates. Conversely, as observed in some cases, the increase in bacterial load after the reduction was expected due to the expression of certain stress responsive genes [58]. Nevertheless, the final reduction in bacterial load in maximum cases was assumptive of the gradual utilization of substrates to the limiting concentration by the bacterial population. Several intrinsic factors might also account for such variations in the survival patterns. Moreover, some isolates reached higher final bacterial loads when inoculated in lettuce and other isolates reached higher final bacterial loads when inoculated in spinach. This could be due to the properties of each produce that might either facilitate or suppress the survival of these microorganisms. Finally, all isolates reached higher final bacterial loads when inoculated produce samples were stored at room temperature compared to storing at refrigeration conditions. This could be due to several adaptation mechanisms that bacteria use to survive cold temperatures. The primary cue for low temperature sensing appears to be membrane rigidification. A membrane-associated sensor is activated through the physical change in the packing of fatty acids in the membrane. The sensor thus perceives and transduces the signal to a response regulator, which induces up-regulation of genes involved in the modulation of membrane fluidity. Additionally, cold-responsive mechanisms may also be triggered due to changes in DNA topology caused by changes in temperature [59]

The PCR analysis showed that all survived isolates from the last time points retained the *mcr-1* gene suggesting that this gene did not adversely affect the fitness of the bacterial isolates. Several studies have showed no considerable fitness cost due to acquiring the *mcr-1-* bearing plasmids in *E.coli*. Other studies found that that *mcr-1*-bearing plasmid initially cause a fitness cost, but this cost was largely reduced after serial cultures. [60]

Disc Diffusion Susceptibility test revealed that the tested isolates from the last time points exhibited minor or no changes in their AMR profiles compared to those of the original isolates suggesting that under stressful conditions, bacteria might have lost some resistance genes. As for the new resistant profiles recorded for the survived isolates that were not part of the AMR profiles of the original isolates, this could be due to the artifacts of the disc diffusion method.

The results obtained imply that the mobile colistin gene; *mcr-1* persists under conditions that are not optimal for the bacterium and that the only way to limit the spread and dissemination of the mobile colistin resistance gene is through banning colistin use in agriculture and food producing animals and limiting its use to therapeutic purposes.

This study is the first to evaluate the survival of multidrug-resistant *mcr-1*-harboring *Escherichia coli* in two of the most commonly consumed raw vegetables, lettuce and spinach under room temperature and refrigeration conditions.

The results obtained emphasize the persistence of the mobile colistin resistance gene and the urge to act to ban colistin use as a prophylactic and growth promoting agent. This also implies that refrigerating fresh produce before selling it does not affect the persistence of the *mcr-1* gene hence the necessity to properly wash vegetables that are consumed raw to minimize the risk of microbial contamination. Thus, several measures should be considered to limit the spread of antimicrobial resistance and colistin resistance in particular. Cooperation to achieve these measures is necessary through joining efforts form different disciplines with the effective contribution of medical doctors, veterinary surgeons, and environment experts. [53]

CHAPTER V

CONCLUSION

Contamination of fresh produce with multidrug resistant bacteria is increasing with the continuous increase of production to cater for the equally increasing demand. Raw consumed vegetables including lettuce and spinach have been reported as major sources of food-borne pathogens due to their microbial contamination. Several sources of contamination of fresh produce have been recognized including soil, irrigation water, wild and domestic animals, insects, and human handling and processing. Antimicrobial resistance is a global concern to public health rendering antibiotics ineffective in treating sever bacterial infections. Another issue facing public health is the increased resistance of Gram-negative bacteria to the last resort antibiotic; Colistin. The mobile colistin resistance gene *mcr-1* has been emerging and persisting in several niches including vegetables rising the issue of its transmission to humans through the food chain. In this study, survival of multidrug *mcr-1*-harboring *Escherichia coli* on lettuce and spinach was reported. The *E.coli* used in this study were previously isolated from sewage, poultry, pigeon fecal matter, and river water. The findings emphasize a major challenge that requires immediate act to limit colistin resistance and prevent the global dissemination of *mcr-1*. Limiting the use of antibiotics to therapeutical purposes and banning the use of colistin in food production are thus critical measures to prevent the escalation of the AMR problem and the spread of *mcr-1* to humans.

REFERENCES

[1] Ventola C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. P & T: a peer-reviewed journal for formulary management, 40(4), 277–283.

[2] Salameh, P., Sacre, H., Hallit, S., & Hajj, A. (2017). ANTIBIOTIC RESISTANCE IN LEBANON. AMR CONTROL.

[3] Osman, M., Mir, H. A., Rafei, R., Dabboussi, F., Madec, J., Haenni, M., & Hamze, M. (2019). Epidemiology of antimicrobial resistance in Lebanese extra-hospital settings: An overview. Journal of Global Antimicrobial Resistance, 17, 123-129. doi:10.1016/j.jgar.2018.11.019

[4] Jammoul, A., & El Darra, N. (2019). Evaluation of Antibiotics Residues in Chicken Meat Samples in Lebanon. *Antibiotics, 8*(2), 69. doi:10.3390/antibiotics8020069

[5] Mokh, S., El Hawari, K., Abdul Rahim, H., Al Iskandarani, M., & Jaber, F. (2020). Antimicrobial residues survey by LC-MS in food-producing animals in Lebanon. *Food Additives & Contaminants: Part B, 13*. doi:DOI: 10.1080/19393210.2020.1739148

[6] Kassaify, Z., Abi Khalil, P., & Sleiman, F. (2013). Quantification of Antibiotic Residues and Determination of Antimicrobial Resistance Profiles of Microorganisms Isolated from Bovine Milk in Lebanon. *Food and Nutrition Sciences, 4*. doi:doi: 10.4236/fns.2013.47A001.

[7] Sourenian, T., Mann, D., Li, S., Deng, X., Jaafar, H., & Kassem, I. I. (2020). Dissemination of multidrug-resistant *Escherichia coli* harboring the mobile colistin resistance gene mcr-1.1 on transmissible plasmids in the Mediterranean

Sea. *Journal of Global Antimicrobial Resistance, 22*, 84-86. doi:10.1016/j.jgar.2020.05.007

[8] Hmede, Z., Sulaiman, A. A., Jaafar, H., & Kassem, I. I. (2019). Emergence of plasmid-borne colistin resistance gene mcr-1 in multidrug-resistant *Escherichia coli* isolated from irrigation water in Lebanon. *International Journal of Antimicrobial Agents, 54*(1), 102-104. doi:10.1016/j.ijantimicag.2019.05.005

[9] Hassan J, Eddine RZ, Mann D, Li S, Deng X, Saoud IP, Kassem II. The Mobile Colistin Resistance Gene, *mcr-1.1*, Is Carried on IncX4 Plasmids in Multidrug Resistant *E. coli* Isolated from Rainbow Trout Aquaculture. *Microorganisms*. 2020; 8(11):1636.

[10] Harakeh, S., Yassine, H., & El-Fadel, M. (2006). Antimicrobial-resistant patterns of Escherichia coli and Salmonella strains in the aquatic Lebanese environments. *Environmental Pollution, 143*(2), 269-277. doi:10.1016/j.envpol.2005.11.027

[11] Harakeh, S., Yassine, H., & El-Fadel, M. (2006). Antimicrobial-resistance of Streptococcus pneumoniae isolated from the Lebanese environment. *Marine Environmental Research, 62*(3), 181-193. doi:10.1016/j.marenvres.2006.03.010

[12] Harakeh, S., Yassine, H., Hajjar, S., & El-Fadel, M. (2006). Isolates of Staphylococcus aureus and saprophyticus resistant to antimicrobials isolated from the Lebanese aquatic environment. *Marine Pollution Bulletin, 52*(8), 912-919. doi:10.1016/j.marpolbul.2005.12.008

[13] Faour-Klingbeil, D., Kuri, V., Fadlallah, S., & Matar, G. M. (2016). Prevalence of antimicrobial-resistant Escherichia coli from raw vegetables in Lebanon. The

Journal of Infection in Developing Countries, 10(04), 354-362. https://doi.org/10.3855/jidc.7745

[14] Saleh, I., Zouhairi, O., Alwan, N., Hawi, A., Barbour, E., & Harakeh, S. (2009). Antimicrobial resistance and pathogenicity ofEscherichia coliisolated from common dairy products in the Lebanon. *Annals of Tropical Medicine & Parasitology, 103*(1), 39-52. doi:10.1179/136485909x384965

[15] Zouhairi O, Saleh I, Alwan N, Toufeili I, Barbour E, Harakeh S. Antimicrobial resistance of Staphylococcus species isolated from Lebanese dairy-based products. East Mediterr Health J. 2012 Dec 4;16(12):1221-5. doi: 10.26719/2010.16.12.1221. PMID: 24988395.

[16] Kabrite S, Bou-Mitri C, El Hayek Fares J, Hassan HF, Matar Boumosleh J (2019) Identification and dietary exposure assessment of tetracycline and penicillin residues in fluid milk, yogurt, and labneh: A cross-sectional study in Lebanon, *Veterinary World*, 12(4): 527-534

[17] Alwan, N., Saleh, I., Beydoun, E., Barbour, E., Ghosn, N., & Harakeh, S. (2010). Resistance ofBrucella abortusisolated from Lebanese dairy-based food products against commonly used antimicrobials. *Dairy Science & Technology, 90*(5), 579-588. doi:10.1051/dst/2010017

[18] Harakeh, S., Saleh, I., Zouhairi, O., Baydoun, E., Barbour, E., & Alwan, N. (2009). Antimicrobial resistance of Listeria monocytogenes isolated from dairy-based food products. *Science of The Total Environment, 407*(13), 4022-4027. doi:10.1016/j.scitotenv.2009.04.010

[19] Saleh, I., Barbour, E., Shaib, H., & Harakeh, S. (2012). Highly resistant Yersinia enterocolitic a isolated from dairy based foods in Lebanon. *The International Arabic Journal of Antimicrobial Agents, 2*. doi:10.3823/706

[20] Harakeh, S., Yassine, H., Gharios, M., Barbour, E., Hajjar, S., El-Fadel, M., . . . Tannous, R. (2005). Isolation, molecular characterization and antimicrobial resistance patterns of Salmonella and Escherichia coli isolates from meat-based fast food in Lebanon. *Science of The Total Environment, 341*(1-3), 33-44. doi:10.1016/j.scitotenv.2004.09.025

[21] Kassem, I. I., Nasser, N. A., & Salibi, J. (2020). Prevalence and Loads of Fecal Pollution Indicators and the Antibiotic Resistance Phenotypes of Escherichia coli in Raw Minced Beef in Lebanon. *Foods, 9*(11), 1543. doi:10.3390/foods9111543

[22] Fadlallah, S.M., Hajj, R.E., Hage, J.E., Nasser, Z., Ghosn, N., Ammar, W., & Matar, G. (2018). Molecular Epidemiology and Antimicrobial Resistance of Campylobacter coli from Caeca and Carcass of Poultry in Lebanon

[23] Dandachi, I., Sokhn, E. S., Dahdouh, E. A., Azar, E., El-Bazzal, B., Rolain, J., & Daoud, Z. (2018). Prevalence and Characterization of Multi-Drug-Resistant Gram-Negative Bacilli Isolated From Lebanese Poultry: A Nationwide Study. *Frontiers in Microbiology, 9*. doi:10.3389/fmicb.2018.00550

[24] Hmede, Z., & Kassem, I. I. (2018). The Colistin Resistance Gene mcr-1 Is Prevalent in Commensal *Escherichia coli* Isolated from Preharvest Poultry in Lebanon. *Antimicrobial Agents and Chemotherapy, 62*(11). doi:10.1128/aac.01304-18

[25] Bialvaei, A. Z., & Kafil, H. S. (2015). Colistin, mechanisms and prevalence of resistance. Current Medical Research and Opinion, 31(4), 707-721. doi:10.1185/03007995.2015.1018989

[26] Wang, R., van Dorp, L., Shaw, L. P., Bradley, P., Wang, Q., Wang, X., ... & Balloux, F. (2018). The global distribution and spread of the mobilized colistin resistance gene mcr-1. *Nature communications*, *9*(1), 1-9.

[27] Chelaghma, W., Loucif, L., Bendahou, M., & Rolain, J. M. (2021). Vegetables and Fruit as a Reservoir of β-Lactam and Colistin-Resistant Gram-Negative Bacteria: A Review. *Microorganisms*, *9*(12), 2534.

[28] Sabala, R. F., Usui, M., Tamura, Y., Abd-Elghany, S. M., Sallam, K. I., & Elgazzar, M. M. (2021). Prevalence of colistin-resistant Escherichia coli harbouring mcr-1 in raw beef and ready-to-eat beef products in Egypt. *Food Control*, *119*, 107436.

[29] Manageiro, V., Jones-Dias, D., Ferreira, E., & Caniça, M. (2020). Plasmid-Mediated Colistin Resistance (mcr-1) in Escherichia coli from Non-Imported Fresh Vegetables for Human Consumption in Portugal. *Microorganisms*, *8*(3), 429.

[30] Liu, B., Li, X., Zhang, Q., Shan, H., Zou, M. & Song, F. (2019). “Colistin-Resistant mcr-Positive Enterobacteriaceae in Fresh Vegetables, an Increasing Infectious Threat in China”. *International Journal of Antimicrobial Agents 54*(1), 89-94.

[31] Zurfuh, K., Poirel, L., Nordmann, P., Nüesch-Inderbinen, M., Hächler, H. & Stephan, R. (2016, March 25). "Occurrence of the Plasmid-Borne mcr-1 Colistin Resistance Gene in Extended-Spectrum-β-Lactamase-Producing Enterobacteriaceae in River Water and Imported Vegetable Samples in Switzerland"; available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4808203/; Internet; accessed November 16, 2020.

[32] Al-Bayssari, C., Dagher, T. N., El Hamoui, S., Fenianos, F., Makdissy, N., Rolain, J. M., & Nasreddine, N. (2021). Carbapenem and colistin-resistant bacteria in North Lebanon: Coexistence of mcr-1 and NDM-4 genes in Escherichia coli. *The Journal of Infection in Developing Countries*, *15*(07), 934-342.

[33] Mikhayel, M., Leclercq, S. O., Sarkis, D. K., & Doublet, B. (2021). Occurrence of the Colistin resistance gene mcr-1 and additional antibiotic resistance genes in ESBL/AmpC-producing Escherichia coli from poultry in Lebanon: A nationwide survey. *Microbiology spectrum*, *9*(2), e00025-21.

[34] Hassan, J., Mann, D., Li, S., Deng, X., & Kassem, I. I. (2021). Emergence of the Mobile Colistin Resistance Gene mcr-1 in Multidrug-Resistant Escherichia coli Isolated from the Fecal Matter of Toddlers in a Community. *Antimicrobial Agents and Chemotherapy*, *65*(5), e00243-21.

[35] Nasser, N. A., Alhaj Sulaiman, A., Mann, D., Li, S., Deng, X., & Kassem, I. I. (2021). Draft Genome Sequences of Multidrug-Resistant and mcr-1.1-Harboring Escherichia coli Isolated from Drinking and Well Waters Used in Syrian Refugee Camps. *Microbiology Resource Announcements*, *10*(2), e01252-20.

[36] Liu RH. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. Am J Clin Nutr. 2003 Sep;78(3 Suppl):517S-520S. doi: 10.1093/ajcn/78.3.517S. PMID: 12936943.

[37] Castro-Rosas J, Cerna-Cortés JF, Méndez-Reyes E, Lopez-Hernandez D, Gómez-Aldapa CA, Estrada-Garcia T. Presence of faecal coliforms, Escherichia coli and diarrheagenic E. coli pathotypes in ready-to-eat salads, from an area where crops are irrigated with untreated sewage water. *Int J Food Microbiology* 2012 May 15;156(2):176-80. doi: 10.1016/j.ijfoodmicro.2012.03.025. Epub 2012 Mar 30. PMID: 22507628.

[38] Luna-Guevara, J. J., Arenas-Hernandez, M., Martínez de la Peña, C., Silva, J. L., & Luna-Guevara, M. L. (2019). The Role of Pathogenic *E. coli* in Fresh Vegetables: Behavior, Contamination Factors, and Preventive Measures. *International journal of microbiology*, *2019*, 2894328. https://doi.org/10.1155/2019/2894328

[39] Jay-Russell MT, Hake AF, Bengson Y, Thiptara A, Nguyen T. Prevalence and characterization of Escherichia coli and Salmonella strains isolated from stray dog and coyote feces in a major leafy greens production region at the United States-Mexico border. PLoS One. 2014 Nov 20;9(11):e113433. doi: 10.1371/journal.pone.0113433. PMID: 25412333; PMCID: PMC4239069.

[40] Food and Drug Administration. (2004). Produce safety from production to consumption: 2004 action plan to minimize foodborne illness associated with fresh produce consumption. *Food and Drug Administration*.

[41] Goodburn, C., & Wallace, C. A. (2013). The microbiological efficacy of decontamination methodologies for fresh produce: a review. Food Control, 32(2), 418-427.

[42] Liu, C., Hofstra, N., & Franz, E. (2013). Impacts of climate change on the microbial safety of pre-harvest leafy green vegetables as indicated by Escherichia coli O157 and Salmonella spp. *International journal of food microbiology*, *163*(2-3), 119–128.

[43] Denis, N., Zhang, H., Leroux, A., Trudel, R., & Bietlot, H. (2016). Prevalence and trends of bacterial contamination in fresh fruits and vegetables sold at retail in Canada. Food control, 67, 225-234.

[44] Stuart D., Shennan C., Brown M. Center for Agroecology and Sustainable Food Systems. Santa Cruz, CA, USA: University of California Santa Cruz; 2006. Food safety versus environmental protection on the Central California coast: exploring the science behind an apparent conflict.

[45] Berger, C. N., Sodha, S. V., Shaw, R. K., Griffin, P. M., Pink, D., Hand, P., & Frankel, G. (2010). Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environmental microbiology*, *12*(9), 2385–2397. https://doi.org/10.1111/j.1462-2920.2010.02297.x

[46] Lynch, M. F., Tauxe, R. V., & Hedberg, C. W. (2009). The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. *Epidemiology and infection*, *137*(3), 307–315. https://doi.org/10.1017/S0950268808001969

[47] Center for Food Safety and Applied Nutrition. (n.d.). *Growth of human pathogens within fruits and vegetables*. U.S. Food and Drug Administration. Retrieved

October 29, 2021, from https://www.fda.gov/food/hazard-analysis-critical-control-point-haccp/potential-infiltration-survival-and-growth-human-pathogens-within-fruits-and-vegetables.

[48] Heaton, J. and Jones, K. (2008), Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: a review. Journal of Applied Microbiology, 104: 613-626. https://doi.org/10.1111/j.1365-2672.2007.03587.x

[49] (CLSI) and the European Committee on Antimicrobial Susceptibility (EUCAST) standards (CLSI, C. (2016). "Performance standards for antimicrobial susceptibility testing". Clinical Lab Standards Institute.

[50] (EUCAST), E.C.o.A.S.T. Recommendations for MIC determination of colistin (polymyxin E) as recommended by the joint CLSI-EUCAST Polymyxin Breakpoints.).

[51] Balali, G. I., Yar, D. D., Afua Dela, V. G., & Adjei-Kusi, P. (2020). Microbial contamination, an increasing threat to the consumption of fresh fruits and vegetables in today's world. *International Journal of Microbiology*, 2020.

[52] Ma, F., Xu, S., Tang, Z., Li, Z., & Zhang, L. (2021). Use of antimicrobials in food animals and impact of transmission of antimicrobial resistance on humans. *Biosafety and Health*, *3*(1), 32-38.

[53] Andrade, F. F., Silva, D., Rodrigues, A., & Pina-Vaz, C. (2020). Colistin Update on Its Mechanism of Action and Resistance, Present and Future Challenges. *Microorganisms*, *8*(11), 1716. https://doi.org/10.3390/microorganisms8111716

[54] Song, J., Kim, J., Oh, S. S., & Shin, J. (2021). Multidrug-Resistant Extended-Spectrum β-Lactamase-Producing Escherichia coli Isolated from Vegetable Farm Soil in South Korea. *Microbial Drug Resistance*

[55] Feroz, F., Senjuti, J. D., & Noor, R. (2013). Determination of microbial growth and survival in salad vegetables through in vitro challenge test. *International Journal of Nutrition and Food Sciences*, *2*(6), 312-319.

[56] Solomon, E. B., Yaron, S., & Matthews, K. R. (2002). Transmission of Escherichia coli O157: H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Applied and environmental microbiology*, *68*(1), 397-400.

[57] Noor, R., Murata, M., & Yamada, M. (2009). Oxidative stress as a trigger for growth phase-specific σE-dependent cell lysis in Escherichia coli. *Journal of molecular microbiology and biotechnology*, *17*(4), 177-187.

[58] Nipa, M. N., Mazumdar, R. M., Hasan, M. M., Fakruddin, M. D., Islam, S., Bhuiyan, H. R., & Iqbal, A. (2011). Prevalence of multi drug resistant bacteria on raw salad vegetables sold in major markets of Chittagong city, Bangladesh. *Middle-East Journal of Scientific Research*, *10*(1), 70-77.

[59] Shivaji, S., & Prakash, J. S. (2010). How do bacteria sense and respond to low temperature?. *Archives of microbiology*, *192*(2), 85-95.

[60] Choi, Y., Lee, J. Y., Lee, H., Park, M., Kang, K., Lim, S. K., ... & Ko, K. S. (2020). Comparison of fitness cost and virulence in chromosome-and plasmid-mediated colistin-resistant Escherichia coli. *Frontiers in microbiology*, *11*, 798.