Development of Bacteriophage Cocktail for bio-control of atypical *Escherichia coli* O177 strains

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SUMMARY

Atypical enteropathogenic *Escherichia coli* (aEPEC) strains are emerging pathogens responsible for deadly diarrhoea infections in human in both developing and industrialised countries across the globe. The aEPEC is a heterogeneous group, which shares virulence traits with other *E. coli* pathotypes from diarrhoeagenic *E. coli* and extraintestinal *E. coli* pathogenic groups. In addition, food-producing animals such as cattle are considered as the primary reservoir of aEPEC strain and thus, this may increase the possibilities of food contamination during milking or at slaughter. Although several interventions have been implemented to combat food contamination, some of the strategies have serious side effects, especially in humans. Furthermore, lack of a novel antimicrobial agents coupled with antibiotic resistance precipitate transmission of antibiotic resistant foodborne pathogens from animals to humans via consumption of contaminated food. This warrant a need to search for a novel and practical intervention such as the use of bacteriophages to curb antimicrobial resistance. Therefore, the purpose of this study was to develop phage cocktails to control *E. coli* O177 strain in food and live animals using *in vitro* models.

Faecal samples were collected from cattle from different farming systems for isolation of *E. coli* O177 strain and *E. coli* O177-specific bacteriophages. In addition, genotypic typing and whole genome sequence techniques were employed to determine genetic similarities and genome features of the *E. coli* O177 isolates. Furthermore, *E. coli* O177-specific phages were isolated using *E. coli* O177 host. Phage morphotype, stability against various physical parameters (pH and temperature were assessed) were assessed. In addition, individual phages and phage cocktails were assessed to determine their effectiveness in reducing *E. coli* O177 cells on artificially contaminated beef and their ability to prevent and destroy pre-formed biofilm structures. Individual phages and phage cocktails were also evaluated for their

effectiveness in reducing *E. coli* O177 cell count in rumen simulation model and complete genome sequence was performed to assess the feature of the phages.

A total of 780 cattle faecal samples were collected from different farms. One thousand two hundred and sevety-two presumptive isolates were obtained. Nine hundred and fifteen of the isolates were successfully identified as *E. coli* isolates through amplification of the *uid*A genus-specific gene. Out of 915 isolates screened, 376 were confirmed as *E. coli* O177 strain using multiplex PCR, targeting the *rmlB* and *wzy* genes. *E. coli* O177 isolates harboured *hlyA, stx*$_2$, *stx*$_1$, *eaeA, stx*$_{2a}$ and *stx*$_{2d}$ (12.74, 11.20, 9.07, 7.25, 2.60 and 0.63%, respectively). Some isolates possessed a combination of the *stx*$_1$/*stx*$_2$/*hlyA*/*eaeA* and one isolate carried the *stx*$_1$/*stx*$_2$/*hlyA*/*eaeA*/*stx*$_{2a}$/*stx*$_{2d}$ genes simultaneously. Furthermore, this study revealed that *E. coli* O177 isolates were resistant to erythromycin, ampicillin, tetracycline, streptomycin, kanamycin, chloramphenicol and norfloxacin (63.84, 21.54, 13.37, 17.01, 2.42, 1.97 and 1.40%, respectively). In addition, 20.7% of the isolates exhibited different phenotypic multi-drug resistance patterns. The Multiple Antibiotic Resistance (MAR) index ranged from 0.29 to 0.86 whereas the average MAR index was 0.65. All 73 isolates harboured at least one antimicrobial resistance gene. The *aadA*, *streA*, *streB*, *erm* and *tetA* resistance genes were detected separately and/or concurrently.

Genetic typing techniques showed 100% genetic similarities of *E. coli* O177 isolates obtained from cattle from different farms. Enterobacterial repetitive intergenic consensus (ERIC) typing clustered the isolates into nine clusters made up of mixed isolates from different farms while Random amplified polymorphism deoxyribonucleic acid (RAPD) typing classified the isolates into eight clusters composed of isolates from the various cattle farms. Whole genome sequence (WGS) annotation indicated that the two genomes sequenced showed > 95% similarities to

O177 strain with H7 (CF-154) and H21 (CF-335). WGS revealed that *E. coli* O 177 genomes contained several virulence and antimicrobial resistance genes sequences. The key virulence genes such as intimin (*eaeA*), haemolysin (*hlyA* and *hlyE*) and others, associated with aEPEC group, were found in both genomes. However, genes (*stx*) encoding for shiga toxins were not found in both genomes. Furthermore, *E. coli* O 177 genomes possessed six plasmid types, prophages and a cluster of regularly interspaced short palindromic repeats (CRISPR) type I (subtype I-A and I-E) gene sequences. The CRISPR-Cas proteins were found in both genomes.

A total of 31 lytic *E. coli* O177-specific bacteriophages were sucessfully isolated in this study. The spot test revealed that all eight selected phages were capable of infecting different environmental *E. coli* strains. In addition, Efficiency of plating (EOP) analysis (range: 0.1 to 1.0) showed that phages were capable of infecting a wide range of *E. coli* isolates. Selected phage isolates had similar morphotype (icosahedral head and contractile tail ranging from 81.2 nm to 110.77 nm and 115.55 nm to 132.57 nm, respectively) and were classified under the order Caudovirales, *Myoviridae* family. The phages were stable at 37 °C and 40 °C, over 60 minutes of incubation. Furthermore, phages were inactive at pH 3.0. However, quadratic response showed that pH optima ranged between 7.6 and 8.0. Phage latent period ranged from 15 to 25 minutes while burst size ranged from 91 to 522 virion particles (PFU) per infected cell.

The current study also showed that eight individual phages and six phage cocktails were capable of reducing *E. coli* O177 cell count on artificially contaminated beef over a seven-day incubation period at 4 °C. Two individual phages, vB_EcoM_12A1 and vB_EcoM_3A1 and three cocktails, T3, T4 and T6, reduced bacteria cell count to below detection limit (1.0 $\log_{10}$ CFU/mL) over a seven-day incubation period. Relative reduction percentage ranged between

73-100% and 32-100% (for all individual phages and cocktails, respectively). Although *E. coli* cell counts showed increase at day three and seven in samples treated with individual phages (vB_EcoM_10C2, vB_EcoM_10C3, vB_EcoM_118, vB_EcoM_11B, vB_EcoM_366B, vB_EcoM_366V) and phage cocktails (T1, T2 and T5), viable cell counts were significantly lower than the controls. Individual phages and phage cocktails also revealed potential in inhibiting the growth of *E. coli* O177 biofilm formation with the later showing greater potency in destroying pre-formed biofilm than the former. This finding suggests that phages cocktails developed in this study can be used for biocontrol of *E. coli* O177 on meat at storage conditions to improve food safety.

Response surface regression analysis revealed significant quadratic responses in the titres of both individual phages and their cocktails over the 48-hour incubation period under simulated rumen fermentation conditions. Individual phage titres were predicted to peak at 50 - 52 hours of *in vitro* ruminal incubation from response surface regression models with R^2 values ranging from 0.811 to 0.994 while phage cocktail titres were predicted to peak at 51 and 55 hours of *in vitro* ruminal incubation from response surface regression models with R^2 values ranging from 0.982 to 0.995. When exposed to individual phages, the percent reduction of *E. coli* O177 cell counts peaked (60.81 - 63.27%) at 47 to 48 hours of incubation as determined from prediction equations with R^2 values ranging from 0.992 to 0.996. Nevertheless, when treated with phage cocktails, the percentage reduction of *E. coli* O177 cell counts peaked (63.06 to 73.25%) at 43 to 46 hours of incubation as determined from prediction equations with R^2 values ranging from 0.970 to 0.993. Over the 48-hour of incubation period, individual phages vB_EcoM_366B and vB_EcoM_3A1 were the most effective (62.31 and 62.74%, respectively) while phage cocktails T1, T3, T4 were the most effective (66.67, 66.92 and 66.42%, respectively) in reducing *E. coli* O177 cell count at 39 °C, over a 48-hour incubation period. These results

indicate that phage cocktail T3, T4, and T6 are the most effective in reducing *E. coli* O177 cell counts in a simulated ruminal fermentation system. Therefore, these phage cocktails are the most suitable candidates to be used in live animals, particularly cattle, to reduce the level of *E. coli* O177 load before slaughter.

Whole genome sequence annotation showed that *Escherichia* phage vB_EcoM_11B2-MVA genome was 152,234 bp linear dsDNA with 39.1% GC content. *Escherichia* phage vB_EcoM_11B2-MVA genome did not contain lysogenic (integrase), virulence or antimicrobial resistance sequences. This indicated that the phage vB_EcoM_11B2 is safe and suitable candidate to be used as a biocontrol agent against the *E. coli* O177 strain either in food or live animals. In addition, the genome contained 30 genes encoding for phage proteins and 11 tRNA gene sequences coding for 10 amino acids. Based on blast pairwise alignment, phylogenomic and VICTOR analysis, *Escherichia* phage vB_EcoM_11B2-MVA genome was classified under the order Caudovirales, *Myoviridae* family and the new genus "*Phapecoctavirus*".

***Keywords*:** Atypical enteropathogenic *E. coli* O177 strain, bacteriophages, biocontrol, cattle, food.

DECLARATION

I, **Montso Kotsoana Peter**, declare that the thesis entitled "**Development of bacteriophage cocktail for bio-control of atypical *Escherichia coli* O177 strains**", submitted for the degree of Doctor of Philosophy in Biology (Molecular Microbiology) at the North-West University and the work contained herein is my own work in design and execution and has not previously, in its entirety or part, been submitted to this or other university for a degree. I further declare that all the materials contained herein have been duly acknowledged.

Candidate: Montso Kotsoana Peter

Signed…… Date……

Promoter: Professor Collins Njie Ateba

Signed…… Date……

Co-promoter: Professor Victor Mlambo

Signed…… Date……

DEDICATION

I dedicate this thesis to my mother, Mrs Mary Mamojabeng Montso who nurtured me. I am dearly grateful ‘m’e.

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PUBLICATIONS FROM THIS THESIS

Montso, P. K., Mlambo, V., Ateba, C. N. 2019. The first isolation and molecular characterization of shiga toxin-producing virulent multi-drug resistant atypical enteropathogenic *Escherichia coli* O177 serogroup from South African cattle. *Frontiers in Cellular and Infection Microbiology*, 9, 333. https://doi.org/10.3389/fcimb.2019.00333

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MANUSCRIPTS UNDER PEER – REVIEW

Montso, K.P., Mlambo, V. and Ateba, C.N., 2019. Evaluation of the efficacy of novel phage cocktails in reducing *Escherichia coli* O177 on artificially contaminated beef and their effectiveness in preventing and destructing biofilm formation. *Journal of Food Microbiology* (**Under review**).

Montso, K.P., Mlambo, V. and Ateba, C.N., 2019. Genetic relatedness and whole genome sequencing of *Escherichia coli* O177 strain isolated from cattle faeces. *Journal Environment International* (**Under review**).

DEFINITION OF CONCEPTS

Antimicrobial resistance: The ability of bacteria to resist and escape from the effects of an antibiotic that was once effective in killing the bacteria.

Bacteriophages: Are bacterial viruses that only infect and replicate within their specific host.

Biofilm: Surface-associated microbial cells that are embedded in a self-produced extracellular polymeric substance matrix.

Biocontrol: The practice or processes by which an undesirable organism is controlled by means of another organism.

Broad host range phage: A phage that infects different hosts within one genus.

Burst size: The ratio of the number of released phage progenies to the infected bacterial host cell.

Contigs: Contiguous sequences assembled from overlapping smaller sequence reads that represent a consensus region of DNA.

Cluster analysis: Comparative analysis of typing data collected for a variety of bacterial isolates in order to group the organism according to their similarity in these data.

Dendrogram: Binary tree illustrating a cluster analysis performed on a number of isolates for any chosen number of typing data.

Fingerprint: A specific pattern (e.g. DNA banding pattern) or set of marker scores displayed by an isolate on application of one or more typing method.

Genome: A complete genetic information of an organism as encoded in its DNA and/or RNA.

Genotype: Genetic constitution of an organism as assessed by a molecular method.

Haemolytic Uraemic syndrome: Clinical symptom characterised by progressive renal failure due associated with microangiopathic haemolytic anaemia, thrombocytopenia.

Host range: The spectrum of strains of bacterial species that a given strain of phage can infect.

Latent period: The time period between phage adsorption and the first release of the phage progeny.

Lineage: A group of species with a common line of descendent from an immediate ancestral species.

Lysogen: A bacterium containing a prophage that is integrated into its genome.

Lytic phage: A phage that upon infection, hijacks host cell mechanisms to produce new phage particles and eventually lyses the host to release the progeny.

Monovalent phage: A phage that recognises a single receptor on the host surface.

Multi-drug resistant: Resistant to at least three or more antimicrobial agent group.

Multiplicity of infection: The ratio of the number of virus particles to the number of target cells present in a defined space.

Next generation sequence: A high-throughput sequencing technology developed in the post-Sanger period.

Pathotype: Group of strains belonging to the same species with a common mode of action with respect to the infection process and virulence.

Phage lytic capability: The ability of a viral particle to lyse bacterial cells.

Phage therapy: Therapeutic use of lytic bacteriophages to treat bacterial pathogen causing infection.

Polyvalent phage: Phage that recognises multiple different receptors on the surface of the host.

Prophage: The latent form of phage DNA that is present in lysogenic bacteria.

Species: A category that circumscribes a genomically coherent group of individual strains sharing high degree of similarity in both genotypic and phenotypic features.

Serotype: Subdivision of a species distinguishable from other strains based on a characteristic set of antigens.

Temperate phage: A phage that integrates its genome into that of its host, forming a lysogen and replicating with the host genome.

Thrombotic thrombocytopenic purpura: Thrombotic thrombocytopenic purpura: is a disorder of the blood-coagulation system, causing microscopic blood to form in the small blood vessels throughout the body.

Typing: Phenotypic and/or genotypic analysis of bacterial isolates below the species or subspecies level performed to generate strain or clone-specific fingerprint or datasets that can be used to detect or rule out cross-infections, elucidate bacterial transmission patterns and find reservoirs or sources of infection in humans.

Virulence factors: Are molecules produced by pathogens that contribute to the pathogenicity of the organism.

Whole genome sequencing: A laboratory process or technique that determines the complete DNA sequence of an organism's genome.

LIST OF ABBREVIATIONS

The following abbreviations have been used throughout this thesis and follow the style recommended by the American Society for Microbiology for Journals.

ATCC	**:**	American Type Culture Collection
CDS	**:**	Coding sequences
CRISPR	**:**	Cluster of regularly interspaced short palindromic
EDTA	**:**	Disodium ethylenediaminetetra-acetic acid
EOP	**:**	Efficiency of plating
ERIC-PCR	**:**	Enterobacterial repetitive intergenic consensus PCR
GRAS	**:**	Generally recognised as safe
HC	**:**	Haemorrhagic colitis
HUS	**:**	Haemolytic uremic syndrome
ICTV	**:**	International Committee on Taxonomy of Virus
LEE	**:**	Locus of Enterocytes Effacement
MLST	**:**	Multilocus sequence typing
MOI	**:**	Multiplicity of infection

NCBI	**:**	National Center for Biotechnology Information
ORF	**:**	Open reading frame
PEG	**:**	Polyethylene glycol
PFGE	**:**	Pulsed field gel electrophoresis
PFU	**:**	Plaque forming unit
RAPD	**:**	Random amplified polymorphism deoxyribonucleic acid
SDS	**:**	Sodium dodecyl sulfate
TEM	**:**	Transmission electron microscopy
TTP	**:**	Thrombotic thrombocytopenic purpura
USADA-FSIS	**:**	United States Department of Agriculture, Food Safety and Inspection Service

CHAPTER ONE

INTRODUCTION AND PROBLEM STATEMENT

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1.1. General introduction

Escherichia coli are versatile and commensal bacterial species that inhabit the gastrointestinal tract of humans and warm-blooded animals (Iwu *et al.*, 2016). These bacteria are commonly found in the lower intestinal tract of their natural hosts. Despite the fact that these organisms are known to occur as normal flora in gastro-intestinal tract of humans and warm-blooded animals, some strains are potentially pathogenic through the expression of a variety of infective and toxin-producing mechanisms, resulting in diseases in humans (Ateba and Mbewe, 2011; Farrokh *et al.*, 2013).

Pathogenic *E. coli* strains possess traits, commonly known as virulence factors, which facilitate the development diseases in their hosts and these include locus of enterocytes effacement (LEE), a variety of toxins and fimbriae or adhesins (Saeedi *et al.*, 2017). Pathogenic *E. coli* strains may be transmitted to humans through the consumption of contaminated food, water and/or contact with contaminated soils (Saeedi *et al.*, 2017; Wang *et al.*, 2017). Shiga toxin-producing *E. coli* strains such O157:H7 cause severe diseases in humans, ranging from uncomplicated diarrhoea to bloody diarrhoea and, in some cases, to the more complicated as well as life-threatening haemolytic uremic syndrome (HUS), haemorrhagic colitis (HC) and thrombotic thrombocytopenic purpura (TTP) (Farrokh *et al.*, 2013; Shen *et al.*, 2015). These complications may be very lethal in infants, young children, the elderly and immuno-compromised individuals (Farrokh *et al.*, 2013; Shen *et al.*, 2015).

While shiga toxin-producing *E. coli* O157 strains have been implicated in most foodborne infections in humans worldwide (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011), in recent years, a number of studies have reported outbreaks of infections in humans caused by non-O157 *E. coli* serogroups (Bielaszewska *et al.*, 2011; Beutin and Martin, 2012; Muniesa *et al.*, 2012; Baranzoni *et al.*, 2014). Non-O157 STEC strains, classified as the "big six", include the serogroups O26, O45, O103, O111, O121 and O145 and they have received a lot of attention due to their involvement in the recent outbreaks in Germany and Japan (Ma *et al.*, 2014; Verhaegen *et al.*, 2016). Most of these outbreaks are usually associated with the consumption of undercooked meat, food products of animal origin, fruits vegetables and/or water that are contaminated with faeces from infected animals (Verhaegen *et al.*, 2016; Kintz *et al.*, 2017). Furthermore, some of the reports have indicated that non-O157 strains cause infections in humans, resulting into life-threatening infections with HUS-like symptoms similar to diseases caused by O157 STEC (Ma *et al.*, 2014; Bai *et al.*, 2015). These non-O157 diseases may result in fatal acute renal failure, which may worsen to become haemolytic anaemia (Kaper *et al.*, 2004). Of particular interest, is the fact that both pathogenic *E. coli* O157 and non-O157 strains are now currently resistant to several antibiotics, commonly used in humans (Ateba and Bezuidenhout, 2008; Ateba *et al.*, 2008; Ahmed and Shimamoto, 2015).

E. coli strains associated with human diseases are broadly grouped into two categories, which are intestinal and intra-intestinal infections (Ombarak *et al.,* 2016). Generally, *E. coli* causing intestinal infections is called diarrhoeagenic *E. coli* (Ombarak *et al.*, 2016). Based on their distinctive virulence properties, pathogenic mechanisms, presence of pathotype-specific genes and clinical symptoms (Kaper *et al.*, 2004), *E. coli* species causing intestinal infections can be further subdivided into six categories such as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) or shiga toxin-producing *E. coli* (STEC),

enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Kaper *et al.*, 2004; Ombarak *et al.*, 2016). In contrast, extra-intestinal infections are grouped into three categories and these include uropathogenic *E. coli* (UPEC), meningitis-associated *E. coli* (MNEC) and necrotoxigenic *E. coli* (NTEC) (Kaper *et al.*, 2004).

Enteropathogenic *E. coli* strains are known as an important causative agent of infant diarrhoeal infections and have also been implicated as a principal cause of diarrhoea in humans within all age groups in both developed and developing countries (Trabulsi *et al.*, 2002; Malik *et al.*, 2017). EPEC is subdivided into two subtypes, namely, typical EPEC (tEPEC) and atypical EPEC (aEPEC) (Tennant *et al.*, 2009). These subtypes are grouped primarily based on the presence or absence of the EPEC adherence factor (EAF) plasmid (Singh *et al.*, 2015; Malik *et al.*, 2017). The tEPEC strains possess a large virulence plasmid known as the EPEC adhesion factor (EAF) plasmid, which encodes for the bundle-forming pili (Bfp) (Malik *et al.*, 2017). The bundle-forming pilus facilitates the adherence of bacterial cell to the intestinal epithelial cells in humans and subsequently, cause diarrhoea (Malik *et al.*, 2017). On the other hand, aEPEC strains possess a type III secretion system encoded in the locus of enterocyte effacement (LEE) but lack virulence factors (*stx* and *bfpA* genes) and therefore, their pathogenic profile is largely unknown (Tennant *et al.*, 2009; Singh *et al.*, 2015; Ingle *et al.*, 2016; Malik *et al.*, 2017). Although the aEPEC are known to be less pathogenic as compared to the tEPEC, recent studies revealed that aEPEC strains have acquired genetic traits that make them pathogenic to humans (Tennant *et al.*, 2009; Ingle *et al.*, 2016). Thus, aEPEC may now have public health implications in humans (Beutin *et al.*, 2005; Tennant *et al.*, 2009; Álvarez-Suárez *et al.*, 2016; Martins *et al.*, 2016).

Domestic animals, particularly cattle, are the principal reservoir of aEPEC such as *E. coli* O177 strain (Wang *et al.*, 2013; Smith *et al.*, 2014; Singh *et al.*, 2015; Malik *et al.*, 2017). In addition, these pathogens are commonly found the in the lower gastrointestinal tract (GIT) in cattle. However, these pathogens, most often, do not result in any symptoms in healthy cattle due to the lack of receptors for potent toxins on the vascular endothelium in their GIT (Saeedi *et al.*, 2017). Therefore, the presence of these organisms in the GIT of cattle does not pose any threat to the animal itself. However, cattle may contribute in the spread of these pathogens by contaminating the environment, water sources, food products and meat with faeces, especially during the slaughtering process (Ateba and Mbewe, 2011; Baker *et al.*, 2016). Against this background, farm management techniques and standard operational procedures have to be strictly implemented at various stages along the food production chain to minimize transmission of pathogens from animals to humans (Ateba and Mbewe, 2011). These may include, but not limited to, the use of probiotics and vaccination as pre-harvest intervention strategies to reduce faecal shedding of pathogenic bacteria especially the non-O157 STEC strains by live animals before slaughter (Smith *et al.*, 2014).

Despite the fact that hygiene standards are practiced along the various stages of the food chain to reduce the level of foodborne contamination, several studies have reported the occurrence of *E. coli* O157 and non-O157 *E. coli* strains in food producing animals, carcasses, food processing facilities and/or abattoirs (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011; Baranzoni *et al.*, 2014; Kumar *et al.*, 2014). Furthermore, these pathogens have been reported even at sales points such as retail outlets; on vegetables, meat and water sources (Ateba and Mbewe, 2011; Abia *et al.*, 2016). In addition, several epidemiological investigations that employed genotyping methods have revealed that there is a correlation between *E. coli* strains isolated from human infections and those that were detected in food products, suggesting a

common source of infection (Ateba and Mbewe, 2013; Ateba and Mbewe, 2014). The situation is even worsened by the fact that multi-drug resistant *E. coli* strains have been isolated from cattle, food products and water intended for humans consumption (Ateba and Bezuidenhout, 2008; Ahmed and Shimamoto, 2015). These findings strongly suggest that current interventions employed to minimise the contamination of food during production are not effective. Given that live animals, especially cattle, harbor foodborne pathogens and also considering the zoonotic nature of *E. coli* strains, there is a need to explore new strategies that will reduce level of pathogenic bacteria on live animals (Rivas *et al.*, 2010). Bacteriophages (also known as phages) are now considered as a promising alternative to reduce faecal shedding of pathogenic bacteria in live animals (Rivas *et al.*, 2010).This intervention is currently considered as a potential approach to enhance food safety (Goodridge and Bisha, 2011; Sillankorva *et al.*, 2012; Bhardwaj *et al.*, 2015). In addition, phages have the potential to control the risks of human exposure to multi-antibiotic resistant foodborne pathogens by reducing their occurrence in live animals (Rivas *et al.*, 2010).

Phages are viruses that infect and subsequently, lyse their host bacteria (Minh *et al.*, 2016). Lytic phages are capable of infecting bacteria cells resulting in cell death due to creation of an imbalance in the osmotic pressure. Phages are host-specific, self-replicating, self-limiting and virtually non-toxic (Waseh *et al.*, 2010; Sillankorva *et al.*, 2012). Interestingly, due to their host specificity, phages do not have any affinity for eukaryotic cells and are therefore, harmless to humans, animals and plants (Sillankorva *et al.*, 2012; Harada *et al.*, 2018). Given that the ubiquitous presence of phages in nature, relatively low costs and ease of isolation, significant progress on characterization novel phages with bio-control potentials in contrast to the development of new antimicrobial agents, phage therapy is not just worth pursuing but provides renewed hope in the fight against bacterial resistance (Sillankorva *et al.*, 2012; Tsonos *et al.*,

2014; Zelasko *et al.*, 2017). Lytic phages have thus been exploited as an efficient tool for various purposes such as improving and promoting environmental and food safety, preventing and/or treating bacterial infections and mitigating foodborne pathogens in live animals (Sillankorva *et al.*, 2012; Sulakvelidze, 2013).

In-vivo studies have revealed promising results on the use of phage cocktails in reducing foodborne pathogens on live animals (O'flynn *et al.*, 2004; Sheng *et al.*, 2006; Rivas *et al.*, 2010). Despite the regulatory processes regarding the use of phages as biocontrol agents, some phage cocktails have been approved and are currently used as natural interventions against bacterial contaminants in food industry (Tan *et al.*, 2014; Perera *et al.*, 2015; Kazi and Annapure, 2016). ListShield, EcoShield, SalmoFresh and Salmonelex are examples of commercial phage products that are currently available in the market (Tan *et al.*, 2014; Perera *et al.*, 2015; Kazi and Annapure, 2016). In addition, phage cocktails have also been used as a pre-harvest intervention in the veterinary sector to reduce bacterial colonization in live animals before slaughter (Sillankorva *et al.*, 2012). Moreover, phages have also been extensively used as a post-harvest intervention strategy to reduce the level of bacterial contamination in food products such as meat, fresh produce, processed food and in the decontamination of food processing plants (Goodridge and Bisha, 2011; Sillankorva *et al.*, 2012; Bhardwaj *et al.*, 2015). This, therefore, indicates that the importance of studies designed to isolate and determine antimicrobial resistance and virulence profiles of *E. coli* O177 strain from food producing animals and the evaluation of lytic capabilities of endemic phages against these pathogens cannot be overemphasized. Moreover, the development of phage cocktails with increased efficacy against pathogens bacteria may have huge epidemiological importance.

1.2. Problem statement

The increased incidence of antibiotic resistant bacteria has caused substantial morbidity and mortality in humans worldwide (Ahmed and Shimamoto, 2015). Outbreaks of foodborne infections have been reported even in countries with advanced food safety regulations and health care facilities (Scallan *et al.*, 2011). In developed countries like USA, the US Food and Drug Administration (FDA) and Food Safety and Inspection Service (FSIS) agencies have intensified food safety regulations and enforced a zero tolerance policy on the presence of foodborne pathogens in food (Sillankorva *et al.*, 2012; Kase *et al.*, 2015). Despites all these efforts, annual incidences of foodborne infections, particularly in USA, have resulted in 48 million illnesses, 128 000 hospitalizations and 3000 deaths (Scallan *et al.*, 2011). Most of these foodborne infections are usually associated with consumption of food products contaminated with *Campylobacter*, *E. coli*, *Listeria*, and *Salmonella* species, primarily originating from domestic animals such as cattle, pigs and poultry (Scallan *et al.*, 2011). Of particular concern, is the occurrence of antibiotic resistant *E. coli* O157 and non O157 strains in cattle, food of animal origin and/or water intended for human consumption (Da Costa *et al.*, 2013). This incidence has increased morbidity and mortality rates, with a significant impact on infants, the elderly individuals and immunocompromised individuals (Parracho *et al.*, 2012). In addition, health care costs incurred during hospitalisation and treatment of infections caused by antibiotic resistant *E. coli* strains as well as the recall of contaminated food products from the market, have serious impact onto gross domestic products and international trade (Baker *et al.*, 2016). Furthermore, antimicrobial resistance greatly limits treatment options and increases the potential of treatment failure with adverse clinical complications (Da Costa *et al.*, 2013).

In developing countries, particularly in Africa, numerous studies have successfully isolated *E. coli* strains from animals, farm facilities, abattoirs and food processing plants food and/or water

intended for human consumption (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2014; Ahmed and Shimamoto, 2015; Abia *et al.*, 2016; Iwu *et al.*, 2016). In addition, these studies have reported the occurrence of multi-drug resistant *E. coli* O157 and non O157 strains in food producing animals, beef, milk and other food products (Ateba and Bezuidenhout, 2008; Abia *et al.*, 2016; Ombarak *et al.*, 2016; Paudyal *et al.*, 2017).

In South Africa, the antibiotic resistant *E. coli* O157 strain has been isolated from various food products, water sources and food producing animals, particularly in the North West province (Ateba and Bezuidenhout, 2008; Ateba *et al.*, 2008; Ateba and Mbewe, 2013; Ateba and Mbewe, 2014). However, an aEPEC such as *E. coli* O177 strain, which has rare pathogenic properties, has not been extensively studied even in other countries (Ingle *et al.*, 2016). Atypical enteropathogenic *E. coli* O177 strain, having different evolutionary histories from *E. coli* O157 serotype, is a heterogeneous group with limited information available about their fitness, stress responses, virulence and anti-microbial resistant profiles (Trabulsi *et al.*, 2002 Croxen *et al.*, 2013; Ingle *et al.*, 2016). This lack of knowledge and great heterogeneity among these strains increases the complexity of developing new strategies that will reduce their occurrence as bacterial contaminants and thus mitigate the risks associated with food and water diseases caused by these pathogens.

Given that cattle are principal reservoirs of multi-drug resistant *E. coli* strains, presence of aEPEC O177 strain in cattle may pose severe and continuous challenges to food safety. This is worsened by the lack of effective and reliable commercial products that may assist in reducing faecal shedding of *E. coli* strains in live animals (Rivas *et al.*, 2010; Bhardwaj *et al.*, 2015). Even though probiotics and vaccination programs were considered as primary intervention strategies, particularly in live animals, the results were not consistent and thus suggesting that

they are not as effective and reliable when compared to the pre-harvest intervention techniques that may significantly reduce *E. coli* colonisation and its shedding in cattle (Smith *et al.*, 2014). Moreover, the sanitizers currently used as post-harvest bacteria contamination intervention strategies along various processed of food chains have been reported to be toxic and not effective (Kazi and Annapure, 2016). This justifies the need to develop new strategies, especially natural biocontrol agents, which have recently shown to be effective, less expensive, safe, non-toxic and usable to reduce foodborne pathogens, especially in live animals (Goodridge and Bisha, 2011; Sillankorva *et al.*, 2012; Bhardwaj *et al.*, 2015).

Despite the immense data reported, especially on *E. coli* O157:H7 and other non-O157 *E. coli* strains (Ateba and Bezuidenhout, 2008; Tennant *et al.*, 2009; Ateba and Mbewe, 2014; Ahmed and Shimamoto, 2015; Singh *et al.*, 2015; Abia *et al.*, 2016; Ingle *et al.*, 2016; Iwu *et al.*, 2016; Malik *et al.*, 2017), currently the virulence and antibiotic resistance profiles of the *E. coli* O177 strain is largely unknown. The current study was therefore, designed to isolate and characterise *E. coli* O177 strain and their corresponding lytic bacteriophages from cattle faeces. A further objective was to evaluate lytic capabilities of the environmental *E. coli* O177-specific bacteriophages in reducing the level *E. coli* O177 on artificially contaminated beef. The study aim was also expanded by assessing the bio-control potential of individual phages and phage cocktails against inoculated *E. coli* O177 strains in an *in-vitro* model ruminal system. The results obtained from this study were expected to significantly contribute scientific knowledge on the virulence and antimicrobial resistance profiles of *E. coli* O177 strain and contribute to the understanding of the lytic capabilities of the *E. coli* O177-specific phage cocktails as well as their potential for reducing the level of *E. coli* O177 strain in live animals.

1.3. Aim and objectives

1.3.1. Aim

The aim of this study was to isolate *E. coli* O177-specific phages and develop phage cocktails for bio-control of *E. coli* O177 strain.

1.3.2 Objectives

The specific objectives of the study were to:

- isolate *E. coli* O177 strain from cattle and determine the occurrence of virulence and antibiotic resistance genes in the isolates.

- determine the genetic relatedness and complete whole genome sequence profile of the *E. coli* O177 isolates.

- isolate lytic *E. coli* O177 specific bacteriophages from cattle and determine the stability of phages against a variety of physical parameters (pH and temperature).

- evaluate lytic capabilities and efficacy of individual phages and phage cocktails in reducing the concentration *E. coli* O177 cells on artificially contaminated meat.

- assess the activity of individual phages and phage cocktails in preventing and destroying biofilm formation.

- assess the ability of phage cocktails in reducing *E. coli* O177 concentration in an *in-vitro* model ruminal system.

- complete genome sequencing of a novel *Escherichia* phage vB_EcoM_11B2.

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CHAPTER TWO

GENERAL LITERATURE REVIEW

CHAPTER TWO

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2.1. Historical background

Genetically, *E. coli* is a diverse group of enteric bacterial species and it is an essential component of the natural gut micro-flora of warm-blooded animals including humans (Timmons *et al.*, 2016). It was first discovered in human faeces by Theodor Escherich in 1885, who named the species *Bacterium coli commune* (Croxen *et al.*, 2013). Despite the fact that the organism was described using several synonyms by other researchers, the name *Escherichia coli* was officially adopted by the Judicial Commission of the International on Bacterial Nomenclature (Castellani and Chalmers, 1919). Since its discovery, *E. coli* is known as one of the most important model organism and the best studied in science (Kaper *et al.*, 2004; Alteri and Mobley, 2012). Owing to its fast growth, utilisation of diverse substrates and the high plasticity of its genome, *E. coli* has been widely exploited in advancing technology, particularly in the field of genetic engineering (Kaper *et al.*, 2004; Franz *et al.*, 2014).

Escherichia coli strain offers an enormous utility for a wide range of applications such as its use as a very suitable cloning host in recombinant DNA technology, mainly due to its well-characterised genome (Kaper *et al.*, 2004; Baker *et al.*, 2016). In addition, the genome of this bacteria has been very instrumental in processes that were designed to provide a detailed understanding of fundamental genetic and biochemical concepts (Alteri and Mobley, 2012). Although *E. coli* is known to be a laboratory workhorse and non-pathogenic, some strains are highly versatile and are capable of causing a wide range of diseases in humans (Croxen *et al.*, 2013; Timmons *et al.*, 2016). This is attributed largely to the potential of the organism to gain or loss genetic material and thus enabling the organism to be highly diverse and adaptive in

different environments. This explains why *E. coli* strains are capable of acquiring virulence and antimicrobial resistance genes from the environment as well as from other bacterial strains, resulting in possible negative implications to human health (Kaper *et al.*, 2004).

2.2.1. Pathogenic *Escherichia coli*

Pathogenic *E. coli* strains harbour distinctive virulence factors, which enable the bacteria to adapt to new ecological niches and thus cause a wide range of diseases to both animals and humans (Kaper *et al.*, 2004; Farrokh *et al.*, 2013). Moreover, based on the virulence attributes of a wide variety of *E. coli* strains coupled with the severity of clinical signs and symptoms they cause in susceptible hosts, these pathogens are classified into different pathotypes (Kaper *et al.*, 2004). Furthermore, there are conserved regions or sequences within the genome of *E. coli* that are associated with specific pathotypes and, therefore, serve as valid genetic markers to facilitate efficient classification into the different pathotypes (Dobrindt, 2005).

2.2.2. Classification of pathogenic *E. coli* strains

Pathogenic *E. coli* are broadly classified into two major groups, namely intestinal and extra-intestinal strains (Kaper *et al.*, 2004; Ombarak *et al.*, 2016). As indicated earlier, this classification is based on the presence and absence of genomic sequences (genes) attributed to particular pathotypes (Kaper *et al.*, 2004; Dobrindt, 2005). Strains causing intestinal infections, particularly in humans are called diarrhoeagenic *E. coli* (DEC) (Fakih *et al.*, 2017). Based on antigenic differences, mechanisms of pathogenicity and clinical symptoms, DEC strains are grouped into six pathotypes; enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) or shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Kaper *et al.*, 2004; Mirhoseini *et al.*, 2018). On the other hand, strains causing extra-intestinal

infections are grouped into three categories and these include uropathogenic *E. coli* (UPEC), meningitis-associated *E. coli* (MNEC) and necrotoxigenic *E. coli* (NTEC) (Kaper *et al.*, 2004). Although DEC comprises six categories, this review to focuses on only two of them (EHEC and EPEC) that are related to the problem of the study.

2.2.2.1. Enterohaemorrhagic *E. coli*

Enterohaemorrhagic *E. coli* is a subset of shiga toxin producing *E. coli* (Kaper *et al.*, 2004). Strains belonging to this category are characterised by the presence of *stx* operon, which encodes for shiga toxins also known as verocytotoxins (Kaper *et al.*, 2004). This *stx* operon was originally acquired from temperate lambdoid bacteriophages through a transduction process (Farrokh *et al.*, 2013). Thus the acquisition of this operon is known to be the key step in the evolution of EHEC (Kaper *et al.*, 2004). In addition, the detection of the *stx* operon is an ideal target for the differentiation of the STEC strains from the non-STEC strains belonging to other categories (Kaper *et al.*, 2004).

The *stx* operon contains A and B5-subunit protein structures, which function as a cohort in processes that result to apoptosis in the host cell, and thus facilitate the development of cytotoxicity (Kaper *et al.*, 2004; Yang *et al.*, 2015). The B5-pentamer is responsible for binding the holotoxin and glucolipid globotriaosylceramide (Gb3) receptor on the host cell surface (Kaper *et al.*, 2004; Shen *et al.*, 2015; Yang *et al.*, 2015). After binding, A-subunit is delivered into the cytoplasm though the process of endocytosis to initiate enzymatic activity (Kaper *et al.*, 2004; Yang *et al.*, 2015) (Ref). The A-subunit protein inhibits protein synthesis by cleaving ribosomal RNA (28S, rRNA) of adenine 4324 at the N-glycosidic bond, thus preventing the binding of tRNA (Yang *et al.*, 2015; Saeedi *et al.*, 2017). This can lead to cell damage through apoptosis process (Kaper *et al.*, 2004).

Based on their antigenic properties, two major antigenic groups of *stx* (*stx*$_1$ and *stx*$_2$) have been identified and characterised (Kaper *et al.*, 2004; Scheutz *et al.*, 2012). These two groups are further divided into subtypes *stx*$_{1a}$, *stx*$_{1c}$ and *stx*$_{1d}$ for *stx*$_1$ and *stx*$_{2a}$, *stx*$_{2b}$, *stx*$_{2c}$, *stx*$_{2d}$, *stx*$_{2e}$, *stx*$_{2f}$ and *stx*$_{2g}$ for *stx*$_2$ (Scheutz *et al.*, 2012; Farrokh *et al.*, 2013; Baranzoni *et al.*, 2016). The *stx*$_1$ is located in the periplasmic space of the bacterial cell while the *stx*$_2$ is in the extracellular fraction (Shimizu *et al.*, 2009). Furthermore, the A_1 subunit of *stx*$_2$ has a higher affinity for ribosomes and a higher catalytic activity compared to the A_1 of *stx*$_1$ (Basu *et al.*, 2016) and this may explains why *stx*$_2$ is known to be more toxic than *stx*$_1$.

According to scientific literature, *stx*$_2$ is highly prevalent than *stx*$_1$ in STEC strains (Ateba and Mbewe, 2011; Karmali, 2018). This may be attributed to the fact that *stx*$_2$ temperate lambdoid phages outnumber or occur more frequently than the *stx*$_1$ phages in the environment and this enhances easy horizontal transfer of the former (Farrokh *et al.*, 2013; Karmali, 2018). Furthermore, *stx*$_2$ is 400 times more toxic than *stx*$_1$ and is considered as the most potent toxin, which leads to life-threatening complications, particularly in humans (Shimizu *et al.*, 2009; Basu *et al.*, 2016). In addition, *stx*$_2$ subtypes; *stx*$_{2a}$, *stx*$_{2c}$ and *stx*$_{2d}$ are most frequently associated with the development of HC and HUS infections in infected patients (Scheutz *et al.*, 2012; Farrokh *et al.*, 2013). This implies that *E. coli* strains harbouring *stx*$_{2a}$, *stx*$_{2c}$ and *stx*$_{2d}$ either individually or combined, may be more virulent than the *stx*$_1$ or both *stx*$_1$ and *stx*$_2$ strains (Farrokh *et al.*, 2013). Therefore, screening for the presence of these subtypes together with their associated antigenic families, particularly in atypical enteropathogenic *E. coli* O177 strain is imperative.

Other accessory virulence genes, which augment the pathogenicity of *E. coli* strains are *hlyA* and *eaeA* (Kaper *et al.*, 2004). The *hlyA* encodes for the haemolysin protein, which is

responsible for lysing erythrocytes and leukocytes in mammals, including humans and thus leading to anaemic conditions (Kaper *et al.*, 2004; Toro *et al.*, 2017). On the other hand, *eaeA* encodes for the plasmid encoded intimin protein that is located in the chromosomal pathogenicity island known as the locus of enterocyte effacement (LEE) (Trabulsi *et al.*, 2002; Kaper *et al.*, 2004). The intimin protein facilitates the attachment of bacterial cells to the intestinal epithelial cells of the host (Trabulsi *et al.*, 2002).

Escherichia coli O157:H7 is one of the most notable STEC strain with huge clinical significance worldwide (Kaper *et al.*, 2004) (Ref). Since the first outbreak in 1982, *E. coli* O157:H7 serotype has become the most important STEC strain, causing life-threatening complications such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) in humans (Riley *et al.*, 1983; Kaper *et al.*, 2004). Research efforts were shifted to focus and delve on elucidating genetic profiles of shiga toxin producing *E. coli* O157 and this may largely account for the vast data that currently exist worldwide about this pathogen (Kaper *et al.*, 2004). Several studies have, therefore, reported the occurrence of this pathogen in different sources such as food products, water, food producing animals and human stools (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011; Ahmed and Shimamoto, 2015; Abia *et al.*, 2016; Jajarmi *et al.*, 2017). Moreover, detected number of reports have documented the presence of STEC virulence genes (*stx*$_1$, *stx*$_2$, *stx*$_1$ and *stx*$_2$ subtypes, *hlyA* and *eaeA*) in *E. coli* O157 strains from different sources thus confirming their pathogenicity (Mcevoy *et al.*, 2003; Ateba and Mbewe, 2011; Iwu *et al.*, 2016; Cha *et al.*, 2018). Previous studies in the North West province, South Africa also revealed that *stx*$_2$ and *hlyA* genes were the most frequently detected among the *E. coli* O157 isolates (Ateba and Mbewe, 2011). The ability to produce shiga toxins coupled with intimin and haemolysin proteins are the key virulence determinants, which enable

E. coli O157 strains to cause haemorrhagic colitis and haemolytic uraemic syndrome in humans.

Despite the great attention directed towards assessing the pathogenicity of *E. coli* O157 strains worldwide, recent studies have detected other non-O157 STEC strains in food items and food producing animals (Ma *et al.*, 2014; Shen *et al.*, 2015; Iwu *et al.*, 2016; Shridhar *et al.*, 2017; Toro *et al.*, 2017). The most recognised non-O157 STEC strains are O26, O45, O91, O103, O111, O121 and O145 and they are reported as foodborne pathogens (Ma *et al.*, 2014; Verhaegen *et al.*, 2016). Some of these strains (serotypes O26, O103, O111, O121, O45 and O145) are highly pathogenic and they have been associated with fatal and life-threatening diseases that are similar to those caused by *E. coli* O157 strains (Ma *et al.*, 2014). In addition, these non-O157 strains harbour *stx*$_1$, *stx*$_2$ as well as their subtypes (Shen *et al.*, 2015) thus increasing their pathogenicity. Based on the severity of clinical signs and symptomatic infections caused by non-O157 strains coupled with their recent involvement in foodborne complications in humans, these strains are referred to as the "top six" non-O157 STEC (Shridhar *et al.*, 2017). The United States Department of Agriculture and Food Safety (USDAFS) has thus declared both the *E. coli* O157 and non-O157 STEC strains as adulterants if present in food (USADA-FSIS, 2011). Therefore, inclusion of these strains during food safety surveillance is imperative.

2.2.2.2. Enteropathogenic *E. coli*

Enteropathogenic *E. coli* is a diarrhoeagenic group that causes fatal diarrhoea infection in infants, particularly in developing countries (Trabulsi *et al.*, 2002). This group comprises strains belonging to several serotypes such as *E. coli* O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158 (Trabulsi *et al.*, 2002). EPEC strains are

characterised by attaching and effacing (A/E), bundle-forming pilus (BFP), presence of the locus of enterocyte effacement (LEE) (Trabulsi *et al.*, 2002; Kaper *et al.*, 2004). The LEE is a key virulence factor that encodes components of a type three secretion system (T3SS), intimin protein, an intimin receptor and translocated receptor (*Tir*) and all these regulate attachment of *E. coli* cells to intestinal epithelial cells (Trabulsi *et al.*, 2002; Kaper *et al.*, 2004; Martins *et al.*, 2016; Karmali, 2018). Although EPEC strains harbour several key virulence determinants similar to those found in EHEC, organisms in this category lack the *stx* operon (Trabulsi *et al.*, 2002; Nguyen *et al.*, 2006; Croxen *et al.*, 2013). For this reason, the presence and absence of the *stx* operon serves as a valid factor for differentiating EPEC from other STEC producing stains.

Enteropathogenic *E. coli* strains are divided into two subtypes, namely, typical enteropathogenic *E. coli* (tEPEC) and atypical enteropathogenic *E. coli* (aEPEC) (Trabulsi *et al.*, 2002; Kaper *et al.*, 2004; Tennant *et al.*, 2009). These subtypes are grouped based on the presence or absence of the EPEC adherence factor (EAF) plasmid (Singh *et al.*, 2015; Malik *et al.*, 2017). Typical enteropathogenic *E. coli* is characterised by the presence of virulence plasmid known as EPEC adhesion factor plasmid (pEAF), which encodes for the bundle-forming pili (*bfp*) (Trabulsi *et al.*, 2002; Kaper *et al.*, 2004). In contrast, aEPEC lacks the pEAF, *bfp* and *tir* virulence factors (Trabulsi *et al.*, 2002; Kaper *et al.*, 2004; Croxen *et al.*, 2013). The detection of bfp operon is therefore an ideal target to differentiate tEPEC from aEPEC strains (Trabulsi *et al.*, 2002).

Atypical enteropathogenic *E. coli* strain is considered to be an emerging foodborne pathogen (Trabulsi *et al.*, 2002; Nguyen *et al.*, 2006; Ingle *et al.*, 2016). Moreover, scientific literature demonstrates that aEPEC outbreaks are more prevalent than tEPEC, especially in industrialised

countries (Trabulsi *et al.*, 2002; Kaper *et al.*, 2004). Although the virulence profiles of this previously considered none pathogenic group of organisms is not well understood, a study by Ingle *et al.* (2016) recently reported the population structure and virulence profile of aEPEC through whole genome sequencing. Using whole genome sequencing, the study discovered that aEPEC strains contain distinct variants of LEE and non-LEE encoded effectors and, therefore, these findings suggest that this group has evolved through horizontal gene transfer and may now be of great public health importance (Ingle *et al.*, 2016). The same study proved that aEPEC genome may contains *bfp* and *stx* operons (Ingle *et al.*, 2016). In addition, the *ehxA*, stx_1, stx_2 and stx_{2c} genes have been detected in aEPEC O177 strain isolated from cattle and human (Beutin *et al.*, 2005). The presence of these virulence determinants may be attributed to the acquisition of functional LEE operons thus changing the genetic profile of this group of organisms. To the best of our knowledge, besides these two studies, no other study has ever reported occurrence of aEPEC O177 strains as well as their pathogenic potentials, especially in South Africa. The dearth of scientific knowledge on aEPEC O177 suggests the need for thorough research to elucidate the virulence profiles and potential risks these isolates (may) pose to humans in the South African food chain.

2.3. Reservoir of atypical enteropathogenic *E. coli*

Atypical enteropathogenic *E. coli* strains have been isolated from food producing animals such as cattle, sheep and goats and these animal species may serve as reservoirs for this pathogen (Croxen *et al.*, 2013; Álvarez-Suárez *et al.*, 2016). However, it is generally known that cattle are primary reservoir of both the STEC/EHEC and aEPEC strains (Trabulsi *et al.*, 2002; Kaper *et al.*, 2004) . Against this background, aEPEC strains are closely related to STEC/EHEC based on the presence of the LEE-positive operon, suggesting high possibilities for the transfer of genetic materials between these groups (Trabulsi *et al.*, 2002). Moreover, the GIT of cattle

houses a pool of temperate lambdoid phages, which harbour the *stx* operon and this may facilitate horizontal transfer of *stx* phages to aEPEC strains thus creating a new novel STEC strains (Eichhorn *et al.*, 2018; Karmali, 2018). An *in-vitro* study demonstrated that there was a lysogenic conversion of wild-type aEPEC strains to STEC through the acquisition of a *stx*-phage (Eichhorn *et al.*, 2018). Moreover, evidence of the emergence of STEC O26 and O104:H4 strains (originally known as aEPEC and EAEC, respectively), through sequential acquisition of *stx* genes from lambdoid phages (Trabulsi *et al.*, 2002; Muniesa *et al.*, 2012; Karmali, 2018). Currently, these STEC O26 and O104:H4 strains are documented among the most pathogenic STEC strains causing devastating food and waterborne diseases in humans worldwide (Muniesa *et al.*, 2012; Ma *et al.*, 2014). It is also worth noting that *E. coli* O157:H7, which is the most notable STEC strain, originated from an aEPEC O55:H7 precursor, which acquired *stx* genes from lambdoid phages (Dallman *et al.*, 2015; Saeedi *et al.*, 2017; Karmali, 2018). This demonstrates that cattle may serve as a hotbed for the emergence of highly virulent strains. Therefore, aEPEC O177 may acquire *stx* genes from STEC strains and/or stx-encoding temperate phages, thus leading to serious public health concerns.

2.4. Role of ruminant animals in transmission of foodborne pathogens to human

Although most foodborne outbreaks caused by pathogenic *E. coli* strains occur as a result of poor hygiene practices, the main source of contamination with foodborne pathogens are ruminants (Soon *et al.*, 2011). Several studies were able to link most of foodborne outbreaks with faecal contamination from cattle (Ateba and Mbewe, 2014; Álvarez-Suárez *et al.*, 2016). The co-circulation of foodborne pathogens across a wide range of animals, particularly cattle, may facilitate the spread of pathogenic microorganisms to humans (Ateba and Mbewe, 2014). The situation is worsened by the fact that generally colonised cattle are asymptomatic carriers of *E. coli* and they continuously shed the pathogens through faeces to the environment (Saeedi

et al., 2017). As a result, faecal matter may contaminate water sources and food products if proper hygiene practices are not implemented.

Besides this, heavy cross-contamination usually occurs during the slaughtering process, particularly in abattoirs (Ateba and Mbewe, 2011). Food product such as milk may also be contaminated during the milking process (Álvarez-Suárez *et al.*, 2016). Once contaminated, food products, especially undercooked meat and raw milk as well as contaminated water when consumed by susceptible individuals, bacteria cells replicate and produce viable toxins that invade epithelial cells and initiate the establishment of disease (Soon *et al.*, 2011; Sillankorva *et al.*, 2012; Álvarez-Suárez *et al.*, 2016)). Although physical conditions such as pH, temperature, osmotic pressure and nutrient availability may affect the stability and viability of invading *E. coli* cells, these conditions are known to induce global stress responses on the alternate sigma factor (*RpoS*), which transforms the *E. coli* cells to enter the stationary phase (Sarjit *et al.*, 2019). This shows that hostile conditions may not necessarily reduce *E. coli* populations, especially in ruminants, but rather enhance the proliferation of the cells in the host.

2.5.1. Antibiotics and antimicrobial resistance

Antibiotics are secondary metabolites that are synthesised naturally by microorganisms or artificially (semi-synthetic or synthetic) by pharmaceutical industries to inhibit the growth or kill pathogenic microbes (Hudson *et al.*, 2017). Antibiotics are grouped based on their modes of action and/or chemical structure (Hudson *et al.*, 2017). They were discovered serendipitously in 1942 by Sir Alexander Fleming (Hudson *et al.*, 2017; Qiao *et al.*, 2018). Following their discovery, life-threatening diseases became easily treatable, saving millions of lives across the world (Tan *et al.*, 2014; Hudson *et al.*, 2017). This ignited interest for developing new antibiotics, which were meant to be used as therapeutic or sub therapeutic

agents to cure bacterial diseases in animals, plants and humans (Qiao *et al.*, 2018). Since then, antibiotics have played a pivotal role in achieving major advances not only in the pharmaceutical industry but also to transformed modern medicine and surgery, agriculture and livestock industries (Qiao *et al.*, 2018).

Although antibiotics are primarily intended for therapeutic and prophylactic purposes in humans and/or animals, some of them are used as growth promoters, especially in food producing animals with an intention to maximise production (Kim *et al.*, 2018; Qiao *et al.*, 2018). The main groups of antibiotics used in animal husbandry are listed in Table 2.1 (Kuppusamy *et al.*, 2018). The use of antibiotics to maximise production results from the rapid increase in population and the rise in demand for animal products such as meat, eggs and milk as source of protein thus mounting severe pressure on production processes (Mlambo and Mnisi, 2019). As a result, the major emerging national economies such as Brazil, Russia, India, China and South Africa (BRICS) have increased their investment, particularly in intensive livestock production due to international growth to meet demands for both meat and animal protein products (Kim *et al.*, 2018). This may open a gap for misuse of the antibiotics thus requires strict adherence to standard operation procedures.

Table 2.1: Main groups of antibiotic used in animal husbandry.

Group	Examples	Purpose
Aminoglycosides	Spectonomycin	Treatment and control of bacteria pathogens
Beta-lactams	Ampicillin	Treatment, prophylaxis and growth enhancement
Ionophore	Monensin	Increase feed conversion ratio and weight gain
Cephalosporines	Cefquinom	Treatment and control of bacteria pathogens
Fluorochinolones	Marbofloxacin	Treatment and control of bacteria pathogens
Lincosamides	Lincomycin	Treatment and control of bacteria pathogens
Macrolides	Erythromycin	Growth promoter in cattle
Sulphonamides	Sulphamethazine	Disease treatment
Tetracycline	Tetracycline	Growth promoter in cattle

2.5.2. Global trend on the usage of antibiotics in livestock

Despite the fact that the global usage of antibiotics in livestock is fragmented, it is reported that approximately 63 000 - 240 000 tons of antibiotics are used in agriculture each year, particularly in livestock production (Kuppusamy *et al.*, 2018). In addition, over 50% of antibiotic intended for medical use in human health, are used in animal farming (Kuppusamy *et al.*, 2018). It is estimated that 45, 148 and 172 mg of antibiotics are used to produce 1 kg of cattle, chicken and pigs, respectively per year (Kuppusamy *et al.*, 2018).

China is the largest producer and consumer of antibiotics for therapeutic and prophylaxis purposes in human and animals (Qiao *et al.*, 2018). A total of 92 700 tons of antibiotics (inclusive of 36 antibiotics) were produced in China in 2013 and 78 146.1 tons were used in food producing animals (Zhang *et al.*, 2015; Qiao *et al.*, 2018). In USA and Brazil, the amount of antibiotics sold for use in animals in 2011 were estimated at 14 600 and 5 600 tons,

respectively (Kuppusamy *et al.*, 2018). Similarly, the usage of antibiotics in livestock (cattle, pigs and poultry) in Australia, Germany, India, Norway, Sweden and UK was estimated at 932, 1 900, 6, 16 and 308 tons, respectively (Kuppusamy *et al.*, 2018). Although comprehensive data on the usage of antibiotics in animals is not available, particularly in South Africa (Van Den Honert *et al.*, 2018), the use of antibiotics in the BRICS countries is projected to increase by 99% in 2030 with China, Brazil and India contributing 30%, 8% and 4%, respectively (Kim *et al.*, 2018; Qiao *et al.*, 2018). This may promote the emergence and spread of antibiotic resistance, particularly in foodborne pathogens. The situation might be even worse, especially in South Africa, where law enforcement on the use of antibiotics in food producing animals is poorly controlled (Van Den Honert *et al.*, 2018).

2.5.3. Antibiotic resistance and its impact on humans

After discovering the first antibiotic, Sir Alexander Fleming proclaimed that overuse of antibiotics may lead to development of antimicrobial resistance (Hudson *et al.*, 2017). Antibiotic resistance is the ability of the bacteria to resist the effects of an antibiotic that was once effective in killing the organism (Qiao *et al.*, 2018). This phenomenon may occur as a result of alteration of antibiotic binding site through mutation of the pre-existing gene sequence, horizontal gene transfer and/or efflux mediated resistance (Qiao *et al.*, 2018). In addition, over prescription and excessive use of antibiotics has led to the emergence of antibiotic resistance (Kim *et al.*, 2018; Qiao *et al.*, 2018).

Prior exposure to antibiotics is considered to be the main driving force influencing the development of antimicrobial resistance in microorganisms, especially in foodborne pathogens (Tan *et al.*, 2014; Kim *et al.*, 2018). The use of antibiotics as growth promoters in food-producing animals explains why these ruminants are considered a hotspot for antibiotic

resistant determinants. Figure 2.1 outlines the different routes for the dissemination of antibiotic resistant pathogens from farm animals to human and their possible effects to public health (Kuppusamy *et al.*, 2018). The majority of antibiotics used in livestock animals are also utilised in humans for therapeutic purpose, thus resistance to such antibiotics may limit treatment options of human diseases (Yang *et al.*, 2019a). Moreover, antibiotic resistant nosocomial bacteria strains have contributed to medical complications in patients undergoing surgical procedures in hospitals and thus resulting in high mortality rates.

Several studies reported different antibiotic resistance genes in foodborne pathogens such as *Campylobacter*, *E. coli, Staphylococcus aureus* and *Salmonella* species isolated from animals, beef, pork, milk, farm environments, water and human stools (Ateba and Bezuidenhout, 2008; Ahmed *et al.*, 2014; Akindolire *et al.*, 2015; Guo *et al.*, 2019). Antibiotic resistance genes encoding for aminoglycosides, beta-lactams, colistin, erythromycin sulphonamides and tetracycline have been the most frequently identified in foodborne pathogens (Ateba and Bezuidenhout, 2008; Bergeron *et al.*, 2015; Liu *et al.*, 2016). Given an alarming rate of the occurrence of antibiotic resistance determinants in foodborne pathogens coupled with the relatively low rate of the development of new antimicrobial agents for clinical use, the world is heading towards the brink of losing the fight against diseases (Patel *et al.*, 2015). As a result, this poses a serious threat to both human and veterinary medicine. Therefore, there is a need to search for new the novel and practical interventions that can be used to curb this phenomenon.

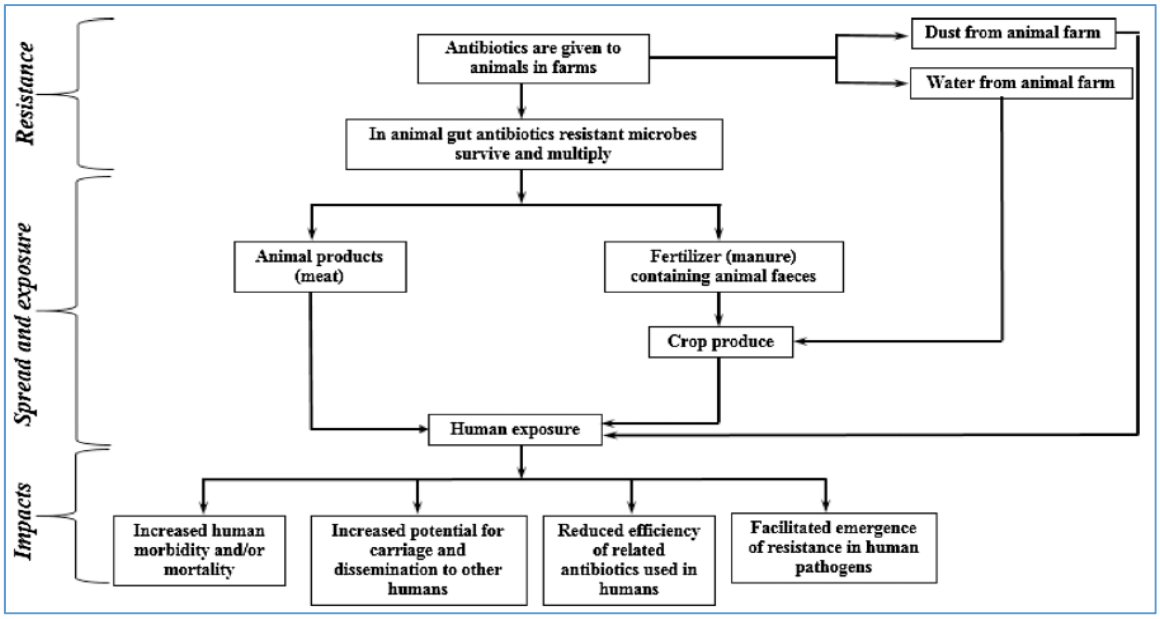

Figure 2.1: Spread of antibiotic resistance from farm animals to human. Adapted from Kuppusamy *et al.* (2018).

2.6. Biofilm formation in food industry

Biofilms are sessile microbial consortia embedded within self-produced polymeric matrix structures attached to hard and moist surfaces (Carpio *et al.*, 2019). Most of foodborne pathogens such as *Campylobacter*, *E. coli*, *Listeria*, *Salmonella* and *Pseudomonas* species are capable of forming biofilms (Endersen *et al.*, 2014; Merino *et al.*, 2019). Biofilm is widely distributed in food industry, hospital equipment and biological structures such as meat, fruits and vegetables (Flemming *et al.*, 2016). The potential to form biofilms presents the bacteria with the capability to resist conventional antimicrobial agents and disinfectants and thus offers protection of microbial cells against hostile conditions such as desiccation, temperature and osmotic stress (Sadekuzzaman *et al.*, 2017; Merino *et al.*, 2019). Biofilms may also cause corrosion on food processing plants resulting in food spoilage and continuous contamination of food along the food chain (Merino *et al.*, 2019). Given that biofilm structures may contain different bacteria species, it can serve as hotbed reservoir for genetic material and thus facilitate the exchange between the same or different bacteria species. The rate of genetic transfer within

the biofilm structures is higher than that of free-living cells due to close contact of the cells (Simões *et al.*, 2010). Genetic transfer and assortment within biofilms may lead to acquisition of new virulence and multi-drug resistance determinants by non-pathogenic strains (Merino *et al.*, 2019). The highly resistant nature of microorganisms in a biofilm to common disinfection procedures, especially those used in food industry indicates the need for employing new control strategies such as bacteriophages to improve food safety from farm to fork (Simões *et al.*, 2010; Merino *et al.*, 2019).

2.7. Food safety intervention

Food is a fundamental daily requirement for humans and it must be safe for consumption (Endersen *et al.*, 2014). However, food safety is continuously challenged by the presence of foodborne pathogens such as *Campylobacter, E. coli, Listeria* and *Salmonella* species in the food chain (Sillankorva *et al.*, 2012). Contamination may occur at any level along the food chain, especially if standard operating procedures are not implemented. Production stages such as those in the farms and abattoirs are considered potential hotspots for microbial contamination (Hungaro *et al.*, 2013). Against this backdrop, several decontamination methods have been developed and clearly included in legislations to ensure sound policy regulations that need to be implemented during food production to curb foodborne contamination from farm-to-fork (Adam and Brülisauer, 2010).

Food processors employ physical, chemical and biological approaches to mitigate food contamination (Hungaro *et al.*, 2013; Sulakvelidze, 2013). Water-based and steam treatment, irradiation, ultrasound, hydrostatic pressure and pulsed electric field chilling are the most common physical intervention strategies used in abattoirs and butcheries to minimize cross-contamination (Hungaro *et al.*, 2013). Chemical intervention procedures include the use of

trisodium phosphate, chlorine and organic acid to reduce microbial contamination (Endersen *et al.*, 2014). Biological approaches employ the use of probiotics and vaccines, particularly on live animals (Smith *et al.*, 2014).

Although these interventions reduce foodborne pathogens in food, their detrimental effects, particularly in humans, is a cause for concern (Hossain *et al.*, 2017). As mentioned earlier, some detergents enhance antibiotic resistance and biofilm formation (Flemming *et al.*, 2016; Merino *et al.*, 2019). Most of the physical and chemical interventions may change the organoleptic properties of food, cause corrosion on food processing plants and toxic effects in humans (Hungaro *et al.*, 2013). This explains why most countries have banned the use of chemicals in food (Adam and Brülisauer, 2010). On the other hand, biological interventions using probiotics and vaccines are very expensive and their candidates are limited to few pathogens. Some of the candidates may contain pathogenic species such as *Enterococcus* and *Bacillus* that usually present health risks to consumers (Hossain *et al.*, 2017). The need for alternative anti-infective agents such as bacteriophages to combat food contamination cannot be over-emphasized.

2.8. Bacteriophages as biocontrol agents

2.8.1. Background on the discovery of bacteriophages

Bacteriophages (known as phages) are viruses that infect and subsequently kill their host bacteria (Chan *et al.*, 2013). In 1896, Ernest Hankin, a British bacteriologist, observed and reported antibacterial activity against *Vibrio cholerae* in water from Ganges and Jumna rivers in India (Sulakvelidze *et al.*, 2001). The report proposed that an unidentified antibacterial agent, which was heat liable and filterable, was responsible for limiting the spread of *Vibrio cholera* and the cholera epidemic within humans (Sulakvelidze *et al.*, 2001). However, Hankin's

findings about the existence of bacteriophages remained dubious, especially in the scientific society (Sharma *et al.*, 2017). In 1898, Gamaleya, a Russian bacteriologist, made a similar observation while working with *Bacillus subtilis* (Sulakvelidze *et al.*, 2001). Despite this, none of these bacteriologists explored their findings further until Frederic Twort, an English bacteriologist observed a similar phenomenon in 1915 (Sulakvelidze *et al.*, 2001; Sharma *et al.*, 2017). Twort hypothesized that an antibacterial activity was caused by an ultra-microscopic virus but he did not pursue his findings further (Sulakvelidze *et al.*, 2001; Endersen *et al.*, 2014).

In 1917, Félix d'Hérelle, a French-Canadian microbiologist at the Institut Pasteur in Paris, officially discovered bacteriophages (Sulakvelidze *et al.*, 2001). His first observation on the phage phenomenon was in 1910 in Mexico when he was investigating an outbreak and developing strategic methods for controlling an epizootic of locust (Sulakvelidze *et al.*, 2001). As part of his investigations, he made preparations of bacterium free filtrates from stool samples of patients with bacillary dysentery infections and incubated the filtrates with a *Shigella* strain isolated from the same patients (Sharma *et al.*, 2017; Kakasis and Panitsa, 2019). An *in-vivo* experiment was performed in which animals were inoculated with a portion of the mixture with an intention to develop a vaccine against bacterial dysentery (Sulakvelidze *et al.*, 2001). Another portion of the mixture was spread-plated on agar plates to observe the growth of the bacteria (Sharma *et al.*, 2017). On the agar cultures, d'Hérelle observed small and clear zones, which he called "*taches*", and later "*taches vieges*" and eventually "*plaques*" (Sulakvelidze *et al.*, 2001; Sharma *et al.*, 2017). He presented his findings at the meeting of Academy of Science in September 1917 (Sulakvelidze *et al.*, 2001) and the work was accepted and published as proceedings of the meeting (D'herelle, 1917). Subsequently, d'Hérelle, together with his wife, proposed the name "bacteriophages", which means "bacteria" and

"phagein" (to eat or devour in Greek) (Sulakvelidze *et al.*, 2001; Sharma *et al.*, 2017). Given that d'Hérelle was the first scientist to present and publish valid evidence of the presence of phages, he was considered to be the discoverer of bacteriophages (Sulakvelidze *et al.*, 2001).

Félix d'Hérelle's publication coupled with him being considered as the one who discovered bacteriophages, stirred up debates among scientists and he was made aware of Hankin and Twort's findings in 1896 and 1915, respectively (Sulakvelidze *et al.*, 2001). However, d'Hérelle argued that the phenomenon described by those scientists, especially Twort was different from his discovery. Meanwhile, d'Hérelle continued his work on bacteriophages and established the idea that bacteriophages were live viruses not "enzymes" as many researchers had thought (Sulakvelidze *et al.*, 2001). Eventually, the priority dispute subsided and the independent discovery of bacteriophages was accepted and was referred to as "Twort-d'Hérelle phenomenon" and later "bacteriophage phenomenon" (Sulakvelidze *et al.*, 2001).

2.8.2. Phage therapy

After his discovery, d'Hérelle made the first attempt to use phages to treat dysentery at the Hôpital des Enfants-Malades in Paris in 1919 (Sulakvelidze *et al.*, 2001). The safety of the phages was tested on d'Hérelle, Hutinel and other hospital interns before administration to a 12-year old patient with severe dysentery (Sulakvelidze *et al.*, 2001). After treatment with a single dose of phages, the patient's symptoms subsided and the patient recovered fully within few days later (Sulakvelidze *et al.*, 2001). Phage efficacy was confirmed after three additional bacterial dysentery patients were treated with a single dose of phages and the patients recovery within 24 hours (Sulakvelidze *et al.*, 2001). However, d'Hérelle did not publish these findings immediately. In 1921, Richard Bruynoghe and Joseph Maisin used bacteriophages to treat staphylococcal skin infections and this is known to be the first reported phage applications in

the treatment of infectious diseases in humans (Bruynoghe and Maisin, 1921). Encouraged by these results, d'Hérell continued his investigations on phage therapy and made various phage preparations to treat cholera infections in humans in India (Summers, 1999).

Although d'Hérell and his counterparts reported therapeutic potential of bacteriophages, phage therapy become obsolete due to the poor understanding of their biology and underlying cause of infectious disease, thus the application of phage therapy was inappropriate (Endersen *et al.*, 2014). This led to various outcomes, which increased skepticisms about phage therapy. In addition, lack of advanced technology to study both the phage and its host, contributed to the fall of phage therapy. Furthermore, phage therapy ceased due to the discovery and commercialization of antibiotics (Endersen *et al.*, 2014). Despite this, phage therapy did not ceased completely in other parts of the world such as in Eastern Europe (Harada *et al.*, 2018).

2.8.3. Phage biology

Bacteriophages are the most ubiquitous entities with an estimate of 10^{31} phage particles on Earth (Hooton *et al.*, 2011; Harada *et al.*, 2018). They are commonly found in all ecosystems where their hosts exist (Sharma *et al.*, 2017) and are diverse in nature, resulting to approximately ten phages that are specific for each bacteria (Hooton *et al.*, 2011; Harada *et al.*, 2018). Bacteriophages lack replication machinery and thus use the genetic material of the host bacteria to reproduce new virions by high-jacking the bacterial cell replication machinery (Tan *et al.*, 2014). Phage infections involve five steps, namely attachment, injection of the viral genome into bacterial host, replication of phage DNA, assembly and release of mature phages (Sulakvelidze *et al.*, 2001). Based on their life cycle, phages are broadly classified into lytic (virulent) and lysogenic (temperate) phages and an overview of these classifications is shown in Figure 2.1.

2.8.4. Lytic life cycle

The lytic cycle begins with the attachment of the phage to specific receptors on the bacterial cell surface (Reece *et al.* 2011). After attachment, a phage injects its genome into the bacterial cell and this is followed by a shutoff of synthesis of the bacterial components thus favouring the synthesis of early phage proteins (Sharma *et al.*, 2017). The replication of phage DNA occurs and then followed by synthesis of late proteins, lysin and holins, that are responsible for bacterial cell lysis. After the assembly process, mature phages lyse the host cell and new phage particles called virions get released to the extracellular environment, Figure 2.1 (Tan *et al.*, 2014). Lytic phages take 20 to 40 minutes to complete their life cycle (Perera *et al.*, 2015; Rao and Lalitha, 2015). This attribute, together with the virulent lifecycle, makes lytic phages preferred candidates for therapeutic purposes.

2.8.5. Lysogenic cycle

Lysogenic phages integrate their genome into bacterial chromosome and remain dormant as prophage, Figure 2.2. As the lysogenic replicates, the phage genome is integrated into the genome of the daughter bacteria cells (Tan *et al.*, 2014). The incorporation of phage DNA into the host may lead to phenotypic changes of the infected bacteria (Sharma *et al.*, 2017), which may enhance bacteria pathogenicity and/or antibiotic resistance (Kakasis and Panitsa, 2019). The phage genome remains suppressed until the excision process is induced by external inducible factors such as UV radiation or antibiotics (Sulakvelidze *et al.*, 2001). This induction process causes temperate phages to enter the lytic life cycle, where the phage replicates, assemble and the viral particles are released through the cell lysis process. Given that the excision prophage sequence from lysogenic bacteria genome is random, adjacent virulence and antimicrobial resistance genes within bacterial genome might be cut together with phage genome. As a result, new phage particles carrying a genome containing both the virulence and

antimicrobial resistance genes may transfer these genes to other bacteria species during phage infection (Farrokh *et al.*, 2013). Therefore, temperate phages are not recommended for therapeutic purpose.

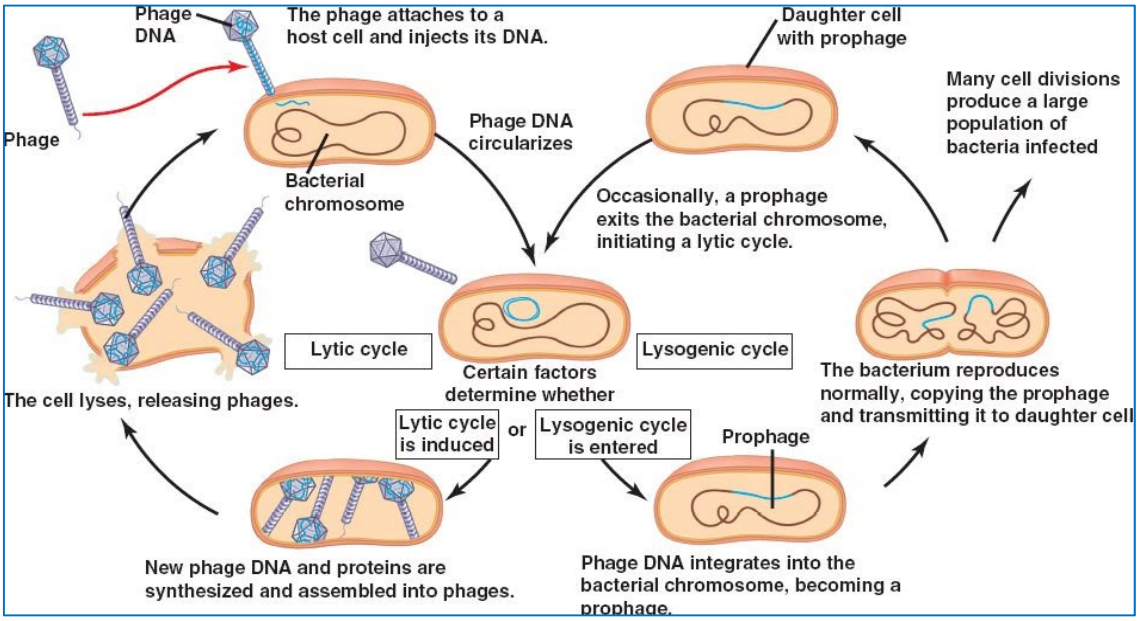

Figure 2.2: Schematic representation of the lytic and lysogenic cycles of phages. Adapted from Reece *et al.* (2011).

2.8.6. Structure and taxonomy of phages

Bacteriophages are diverse in their biological, structural and physicochemical properties (Ackermann, 2007). A typical structure of phage particle comprises a three-dimensional form, that is made up of an icosahedral protein capsid either with or without a tail and a filament, Figure 2.3 (Ackermann, 2007; Ye *et al.*, 2019). The sizes of phage particles range from 24 to 200 nm (Sharma *et al.*, 2017). The *E. coli* infecting phage, T4 is the biggest phage with 200 nm in length and 80-100 nm wide (Sharma *et al.*, 2017; Ye *et al.*, 2019).

The International Committee on Taxonomy of Virus (ICTV) is responsible for classification of bacteriophages (Sharma *et al.*, 2017). In addition, phage classification scheme is based on the phage morphotype and type of nucleic acid, Figure 2.4. Based on these, there are 22 bacterial

and archaeal bacteriophages families (https://talk.ictvonline.org/taxonomy/) (Stone *et al.*, 2019) and the majority (13) of these phage families belonging to the order Caudovirales, Table 2.2 (Ackermann, 2007). Despite the fact that more than 96% of phages are tailed phages that contain dsDNA, they vary with respect to their shapes, filamentous and pleomorphic forms (Kakasis and Panitsa, 2019). Phages belonging to the families *Myoviridae* (long contractile tail), *Siphoviridae* (long non-contractile tail) and *Podoviridae* (short non-contractile tail) are the most common dsDNA phages found in the environment (Harada *et al.*, 2018; Stone *et al.*, 2019).

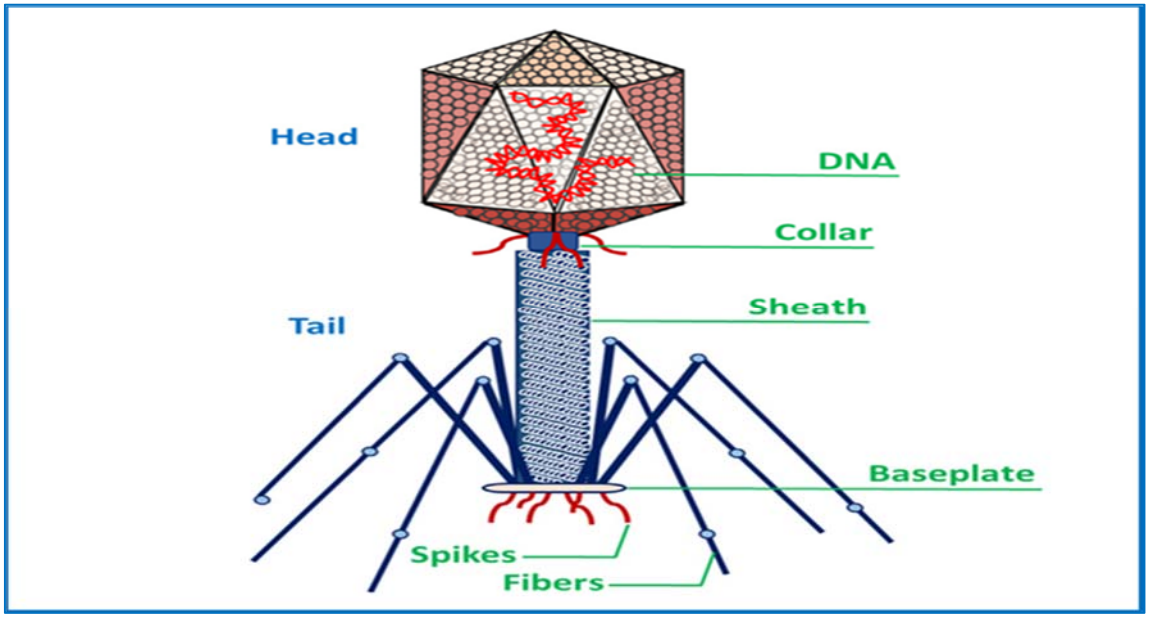

Figure 2.3: Schematic representation of a typical structure of lytic bacteriophages. Adapted from Ye *et al.* (2019).

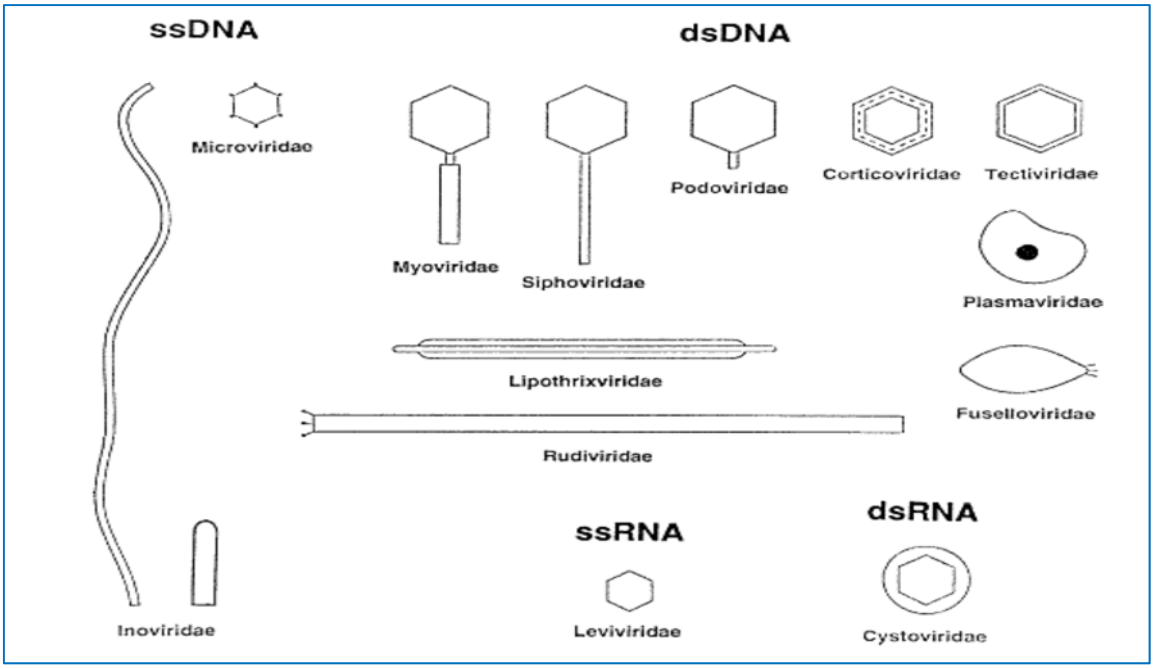

Figure 2.4: Classification of bacteriophages based on their morphotype and genetic material. Adopted from Ackermann (2007).

Table 2.2: Basic properties of phage families.

Nucleic acid	Family name	Characteristics	Examples
Linear dsDNA	*Myoviridae*	Contractile tail	T4
	Siphoviridae	Long, non-contractile tail	λ
	Podoviridae	Short, non-contractile tail	T7
Circular ssDNA	*Microviridae*	Isometric particles without tail	φX174
Circular dsDNA	*Corticoviridae*	Isometric particles without tail	PM2
Linear dsDNA	*Tectiviridae*	Isometric bacteriophages particles without tail	PRD1
Linear ssRNA	*Leviviridae*	Isometric bacteriophages particles without tail	MS2
Enveloped segmented, linear dsRNA	*Cystoviridae*	Spherical bacteriophages particles without tail	φ6
Circular ssDNA	*Inoviridae*	Filamentous bacteriophages particles without tail	fd
Enveloped, circular ssDNA	*Plasmaviridae*	Pleomorphic bacteriophages particles without tail	L2
Linear dsDNA	*Lipothrixviridae*	Enveloped filaments, lipids bacteriophages particles without tail	TTV1
	Rudiviridae	Helical rods bacteriophages particles without tail	SIRV1
Circular dsDNA	*Fuselloviridae*	Enveloped, lipids, no capsid bacteriophages particles without tail	SSV1

2.8.7. Application of bacteriophages in food industry

Food safety is a complex process which requires a comprehensive strategy to prevent contamination from farm-to-fork (Doyle and Erickson, 2012; Endersen *et al.*, 2014). Although several approaches have been implemented to improve food safety, the occurrence of foodborne outbreaks is still relatively high due to bacterial contamination within the food chain (Moye *et al.*, 2018). A rapid increase in the occurrence of multi-drug resistant pathogenic bacteria has put notable and significant pressure on public health practitioners and pharmaceutical companies (Altamirano and Barr, 2019). Conventional food safety measures are not completely infallible, and new approaches such as the use of lytic bacteriophages is increasingly being accepted as a green technology to mitigate foodborne pathogens, especially in food industry (Moye *et al.*, 2018). The efficacy of phages in reducing foodborne pathogens at different stages of the food chain has been documented (Sillankorva *et al.*, 2012; Tan *et al.*, 2014). In industrialised countries, several lytic phages such as ListShield™, EcoShield™, SalmoFresh™ and Salmonelex™ have the "granted generally recognized safe" (GRAS) status for use in food processes to eradicate foodborne pathogens (Endersen *et al.*, 2014; Tan *et al.*, 2014). The use of phages with the aim to promote food safety can be applied at two levels, which include the pre and post-harvest stages along the food chain (Sillankorva *et al.*, 2012; Endersen *et al.*, 2014).

2.8.7.1. Pre-harvest intervention

Considering that foodborne pathogens reside in the gastrointestinal tract (GIT) of farm animals, application of phages on live animals is an ideal approach to prevent the spread of pathogens from contaminating food products, water and/or the environment (Doyle and Erickson, 2012; Sillankorva *et al.*, 2012). In pre-harvest intervention strategies, lytic phages are applied directly on live animals before slaughter (Goodridge and Bisha, 2011; Tan *et al.*, 2014). Several studies

have evaluated and demonstrated the potential of lytic phages in reducing major foodborne pathogens such as *Campylobacter*, *E. coli*, *Listeria* and *Salmonella* species in live animals, using rumen models and/or laboratory animals (Rozema *et al.*, 2009; Rivas *et al.*, 2010; Endersen *et al.*, 2014). Rumen simulation is the most common model used to test the effectiveness of phages in reducing foodborne pathogens in the GIT, particularly ruminants (Goodridge and Bisha, 2011) and this could be attributed to the fact that using live animals for trials requires stringent regulations to be followed due to ethical issues. In addition, the rumen simulation model is relatively easy to monitor and the duration of the experiment is short. *In-vitro* studies have demonstrated the effectiveness of phage cocktails in reducing *E. coli* using the rumen simulation model (Bach *et al.*, 2003; Rivas *et al.*, 2010) during which bacterial reductions were observed between one to two hours of incubation after exposure to phages.

Besides employing the rumen simulation model, laboratory animals such as cattle, pigs and sheep have been used to determine the effectiveness of phages in reducing foodborne pathogens in the GIT (Goodridge and Bisha, 2011). Although the acidic condition and the enzymes in the abomasum present hostile environments for the stability of phages, the application of phages on live animals, particularly via the oral route, revealed promising results in reducing foodborne pathogens (Raya *et al.*, 2006; Callaway *et al.*, 2008; Rozema *et al.*, 2009; Rivas *et al.*, 2010; Raya *et al.*, 2011). However, the application of individual phages in steers and/or sheep showed no significant reduction of *E. coli* O157 when compared to the control (Bach *et al.*, 2003; Rozema *et al.*, 2009; Rivas *et al.*, 2010). This might be due to the complex environment of the GIT (Goodridge and Bisha, 2011). On the other hand, administering phage cocktails in sheep and calves artificially inoculated with *E. coli* O157, revealed a significant reduction of the bacterial cell counts (Raya *et al.*, 2006; Callaway *et al.*, 2008; Rozema *et al.*, 2009; Rivas *et al.*, 2010; Raya *et al.*, 2011). Another study indicated a significant reduction of

Salmonella species in pig using a *Salmonella*-specific phage cocktail (Wall *et al.*, 2010). Although application of phage cocktails via oral route revealed promising results, some studies demonstrated that administering phages via rectal route revealed no significant reduction of the faecal shedding of *E. coli* as compared to the control treatment (Rozema *et al.*, 2009). This could be attributed to the short retention of phages in the rectum compared to the oral route. Furthermore, the procedure of administering phages via the rectal route is not as easy as the oral route. Therefore, this may affect efficacy of the phage, resulting in poor outcomes of the phage therapy.

2.8.7.2. Post-harvest Application

Since the acceptance of the use of phages in food, lytic phages have been widely applied directly onto the surface of food to mitigate foodborne pathogens (Sillankorva *et al.*, 2012; Tan *et al.*, 2014). Lytic phages can be added to food during the packaging process to preserve and extend the shelf life of food products (Sillankorva *et al.*, 2012; Lone *et al.*, 2016). Several studies have demonstrated the effectiveness of different lytic phages (individual phages and/or phage cocktails) in reducing foodborne pathogens (*Campylobacter*, *E, coli*, *Listeria* and *Salmonella* species) on various food products such as raw meat (carcasses), vegetables, dairy products and other processed food (Sillankorva *et al.*, 2012; Endersen *et al.*, 2014; Tan *et al.*, 2014; Snyder *et al.*, 2016). *In-vitro* studies revealed that the application of lytic phage cocktails on artificially contaminated beef and milk could reduce *E. coli* O157 cell counts to below detection limits (O'flynn *et al.*, 2004; Minh *et al.*, 2016; Tomata *et al.*, 2018). The ability of a phage cocktail to reduce Salmonella Typhimurium below detection limits on artificially contaminated pig skin incubated at 4 °C also provides evidence of phages as valuable bio-control agents in post-harvest processes (Hooton *et al.*, 2011). Similarly, *Salmonella* phage cocktails resulted into significant reduction of *Salmonella enteritidis* and *Salmonella*

typhimurium respectively on experimentally contaminated breast chicken and lettuce incubated at 25 °C (Duc *et al.*, 2018; Huang *et al.*, 2018; Bai *et al.*, 2019). Moreover, lytic phages have produced significant reduction of *Listeria monocytogenes* on artificially contaminated vegetables (Sadekuzzaman *et al.*, 2017). Nevertheless, bacteria regrowth after exposure to phages (Duc *et al.*, 2018; Tomata *et al.*, 2018), presents a challenge to post-harvest intervention strategies based on the claim that it may not completely eradicate foodborne pathogens on the food product.

Besides reducing bacterial load, the application of lytic phages as post-harvest intervention agents prevents biofilm formation by foodborne pathogens such as *E. coli*, *Listeria* and *Salmonella* species on food and food processing plants (Endersen *et al.*, 2014). *In-vitro* studies have demonstrated the effectiveness of lytic phages to destroy and/or prevent biofilm formation on raw food (Sadekuzzaman *et al.*, 2017; Yuan *et al.*, 2019). Moreover, phage cocktails have demonstrated broad spectrum activity in destroying established biofilms than their respective individual phages (Endersen *et al.*, 2014). Despite the fact that application of lytic phages as post-harvest intervention have produced promising results in reducing foodborne pathogens on food, the components of the food matrix such as fat, proteins and carbohydrates may also affect the efficacy of phages (Goodridge and Bisha, 2011). In addition, intrinsic properties of food such as pH, moisture content and UV light may inactivate the phages and thus affect phage activity on the host bacteria (Tsonos *et al.*, 2014; Harada *et al.*, 2018). This may seriously limit the suitability of potential lytic phages, particularly if employed as post-harvest intervention candidates to ensure food safety.

2.8.8. Phage therapy hurdle for large scale production and industrial use

Despite the promising results presented by bacteriophages as biocontrol intervention strategies in food industry, there are limitations that may affect the scope of phage therapy (Tsonos *et al.*, 2014). In the context of phage therapy, prior knowledge of the identity of the pathogen (host) at species or strain level is a prerequisite for successful isolation, developing and application of phage therapy (Altamirano and Barr, 2019). This process is tedious, resource demanding and may cause unnecessary delay while the disease is progressing in the patient (Altamirano and Barr, 2019). Furthermore, in the case of non-cultivable pathogens, phage isolation may not be possible at all. As a result, eradication of non-cultivable pathogens using phages, particularly in the food industry, may not be attainable. On other hand, strain and species specificity may affect the efficacy of individual phages, particularly if the food item to be treated is contaminated with two or more unknown different foodborne pathogens (Moye *et al.*, 2018; Altamirano and Barr, 2019). Moreover, the emergence of phage resistant mutants to single phages may hamper the effectiveness of phage therapy (Labrie *et al.*, 2010). Although this can be addressed by employing phage cocktails, preparing phage cocktails requires longer and complex procedures (Altamirano and Barr, 2019).

Given that phages are biological entity, they behave differently due to their genetic diversity and thus, physiochemical characteristics of each phage must be evaluated before such a phage candidate can be included into a cocktail (El-Shibiny and El-Sahhar, 2017; Harada *et al.*, 2018). Furthermore, extra care must be taken to avoid temperate phages for biocontrol purposes since they are capable of integrating their genetic material (DNA) into the host genome and this may promote the transfer of undesirable sequences such virulence and antimicrobial resistance genes to the progeny (Altamirano and Barr, 2019). This may lead to the emergence of novel

pathogenic strains and it is therefore, imperative to determine the safety properties of a potential phage candidate with whole genome sequence analysis as the ideal technique.

Although several phages have GRAS status for use in food, no legal framework on application and vigilance of the use of phage therapy in live animals (Altamirano and Barr, 2019). Pharmaceutical companies are reluctant to start participating and developing phage products for use in phage therapy applications in food producing animals (Altamirano and Barr, 2019)). In addition, determining and establishing the appropriate dosage and route of delivery are very import to ensure effectiveness of phage therapy (Altamirano and Barr, 2019). The route of administration depends on the site and type of bacterial infection (El-Shibiny and El-Sahhar, 2017). However, there is no conventional route for administering phage therapy, particularly in food producing animals, to reduce foodborne pathogens (Altamirano and Barr, 2019). Even though oral administration of phage therapy in live animals is considered as an ideal route towards reduction of foodborne pathogens, different conditions within the GIT of animals affect the effectiveness of the phages, resulting in poor outcomes (Patel *et al.*, 2015). For example, the pH in the abomasum is less than 3.0 and this may affect the stability and infectivity of phages (Callaway *et al.*, 2008; Rozema *et al.*, 2009; Rivas *et al.*, 2010; Raya *et al.*, 2011). There is also no standardized formulation and dose for phage therapy and thus, overdose may increase the chances of inducing immune responses, particularly in live animals (Altamirano and Barr, 2019). These hurdles warrant a need for thorough studies on the phage before approving it in therapeutic applications to ensure safety in live animals.

2.9. Molecular typing of foodborne pathogens

Several molecular typing methods have been developed and extensively employed for typing foodborne pathogens (Ateba and Mbewe, 2014; Adzitey *et al.*, 2013). These include amplified

fragment length polymorphism (AFLP), enterobacterial repetitive intergenic consensus (ERIC), multilocus sequence typing (MLST), pulsed field gel electrophoresis (PFGE) and random amplified polymorphism deoxyribonucleic acid (RAPD) (Foley *et al.*, 2009; Adzitey *et al.*, 2013). These typing tools provide an understanding of genetic similarities and distribution of foodborne pathogens within different sources and/or geographical areas (Nath *et al.*, 2010; Ateba and Mbewe,; 2014). In addition, these typing methods assist in the source tracking or surveillance bacteria contaminants during sporadic or outbreaks of foodborne infections (Adzitey *et al.*, 2013).

The application of molecular typing methods depends on the performance, discriminatory power, reproducibility, typeability and cost effectiveness (Foley *et al.*, 2009; Adzitey *et al.*, 2013). Based on these attributes, PFGE is considered to be the "gold standard" typing method for foodborne pathogens and has produced very high quality data for *Campylobacter*, *E. coli*, *Listeria*, *Salmonella*, *Staphylococcus aureus* species (Foley *et al.*, 2009; Adzitey *et al.*, 2013; Sabat *et al.*, 2013). The method uses restriction enzymes such as *XbaI*, *NotI* and *SmaI* that recognize specific restriction sites within the genomic DNA of the bacteria and cut the molecule into fragments ranging from 20 to 2000 kb that are later resolved by electrophoresis to produce isolate-specific fingerprints (Lopez-Canovas *et al.*, 2019; Tang *et al.*, 2019). The suitability of PFGE as a valuable typing tool is based on its high (≥ 95%) discriminatory power, reproducibility and typeability and its ability to differentiate closely related strains (Foley *et al.*, 2009; Lopez-Canovas *et al.*, 2019).

The AFLP, ERIC, MLST and RAPD are PCR-based typing methods, which despite their low discriminatory power compared to PFGE, are cost-effective and easy to perform (Adzitey *et al.*, 2013). These methods use primer sequences targeting conserved regions (AFLP, ERIC and

RAPD) and housekeeping (three to seven selected conserved) genes for the MLST within the bacterial genome (Adzitey *et al.*, 2013; Tang *et al.*, 2019). The data obtained from MLST typing can be deposited on the internet (http://pubmlst.org and www.mlst.net) and used to compare results among laboratories and/or countries (Adzitey *et al.*, 2013; Sabat *et al.*, 2013). The AFLP, ERIC and RAPD typing methods are relatively cheap, quick and have moderate to high discriminatory power, reproducibility and typeability (Adzitey *et al.*, 2013).

Although the afore-mentioned fingerprinting methods are widely used for genetic typing of different foodborne pathogens for outbreak investigations and source tracking, none of them is not infallible (Adzitey *et al.*, 2013). Generally, no molecular typing method is applicable to non-cultivable microorganisms. In addition, no genetic information such as virulence potential and/or presence of antimicrobial resistance genes can be obtained by these methods (Collineau *et al.*, 2019). Furthermore, small mutations of the targeted gene or restriction sites may result to changes in the amplicon sizes and thus the bands generated may not represent the same sequence of the DNA (Tang *et al.*, 2019). This suggests that genetic relatedness determined by the above methods may not represent a true phylogenetic relationship between the isolates. The PFGE and MLST are costly and require sophisticated equipment and high-level technical expertise for the analysis of data while AFLP, ERIC and RAPD have relatively discriminatory power as compared to the first two methods (Adzitey *et al.*, 2013). These limitations can be solved by the use of next generation sequence (NGS) technique such as whole genome sequence (WGS) approach.

Whole genome sequencing is a sequence based typing method, which detects variants at the single-nucleotide level of the entire genome of an isolate (Tang *et al.*, 2019). Bacterial genomic DNA sequence can be performed on various platforms such as Illumina, Ion Torrent, PacBio

and Nanopore (Jagadeesan *et al.*, 2019). Based on its extremely high discriminatory power, reproducibility and precision for studying the pathogens at genomic level, WGS has revolutionized molecular typing and surveillance of outbreaks of foodborne diseases (Jagadeesan *et al.*, 2019; Rouzeau-Szynalski *et al.*, 2019). This method has the ability to discriminate isolates of the same species and thus refute their epidemiological relationship. Contrary to molecular typing, WGS provides robust information such as virulence potential and antimicrobial resistance genes present in bacterial genome and/or bacteriophages (Rantsiou *et al.*, 2018; Jagadeesan *et al.*, 2019). Furthermore, WGS data can be used to predict and annotate the function of unknown genes present in bacterial genome (Jagadeesan *et al.*, 2019). This suggests that applying WGS in food industry and public health for food safety and disease surveillance may be of great benefit in the development of policies and strategies to mitigate foodborne infections (Jagadeesan *et al.*, 2019; Rouzeau-Szynalski *et al.*, 2019).

Several public health agencies and recognized bodies such as Food and Drug Administration (FDA), Center for Disease Control and prevention (CDC), Public Health England, European Center for Disease Prevention and Control (ECDC) and World Health Organization (WHO) are routinely using WGS for detection and characterisation of foodborne pathogens to support epidemiological investigations (Rantsiou *et al.*, 2018; Jagadeesan *et al.*, 2019). Several foodborne pathogens and bacteriophages have been identified and characterised using WGS (Joensen *et al.*, 2014; Yokoyama *et al.*, 2018; Bai *et al.*, 2019; Yang *et al.*, 2019b). In order to confirm the safety properties of phage candidates for therapeutic purposes, WGS is a prerequisite for providing not just an overview of the genome map but essential to screen for the absence of undesirables gene segments (Yokoyama *et al.*, 2018; Bai *et al.*, 2019). Data derived from WGS of foodborne pathogens and bacteriophages are deposited and stored into the National Center for Biotechnological Information (NCBI) database

(https://www.ncbi.nlm.nih.gov/) and thus provide a platform for the global sharing and comparison of WGS data between laboratories and/or countries (Jagadeesan *et al.*, 2019). Therefore, the use WGS tool cannot be overemphasized.

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CHAPTER THREE

THE FIRST ISOLATION AND MOLECULAR CHARACTERISATION OF SHIGA-TOXIGENIC VIRULENT MULTI-DRUG RESISTANT ATYPICAL ENTEROPATHOGENIC *ESCHERICHIA COLI* O177 SEROGROUP FROM SOUTH AFRICAN CATTLE

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CHAPTER THREE

MOLECULAR CHARACTERISATION OF ATYPICAL ENTEROPATHOGENIC *ESCHERICHIA COLI* O177 STRAIN ISOLATED FROM CATTLE

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Abstract

Atypical enteropathogenic *E. coli* (aEPEC) is a group of diarrhoeagenic *E. coli* with high diversity of serotypes, which lack the bundle-forming pili (BFP) and genes encoding shiga toxins. The aim of this study was to isolate, identify and determine virulence and antibiotic resistance profiles of aEPEC O177 serotypes from cattle faeces. A total of 780 samples were collected from beef and dairy cattle and analysed for the presence of *E. coli* O177. One thousand two hundred and seventy-two (1272) isolates were obtained and 915 were confirmed as *E. coli* species. Three hundred and seventy-six isolates were confirmed as *E. coli* O177 through amplification of *rmlB* and *wzy* gene sequences using multiplex PCR. In addition, none of these isolates harboured *bfpA* gene. A large proportion (12.74%) of the isolates harboured *hlyA* while 11.20%, 9.07%, 7.25%, 2.60% and 0.63% possessed stx_2, stx_1, *eaeA*, stx_{2a} and stx_{2d}, respectively. Most of *E. coli* O177 isolates carried stx_2/*hlyA* (9.74%). Furthermore, 7.4% of the isolates harboured stx_1/stx_2 whereas 7.09% possessed stx_1/stx_2/*hlyA* genes. Only one isolate harboured stx_1/stx_2/*hlyA*/*eaeA*/stx_{2a}/stx_{2d} while 5.11% of the isolates harboured all the four major virulence genes stx_1/stx_2/*hlyA*/*eaeA*, simultaneously. Further analysis revealed that the isolates displayed varied antimicrobial resistance to erythromycin (63.84%), ampicillin

(21.54%), tetracycline (13.37%), streptomycin (17.01%), kanamycin (2.42%), chloramphenicol (1.97%) and norfloxacin (1.40%). Moreover, 20.7% of the isolates exhibited different phenotypic multi-drug resistance patterns. All 73 isolates harboured at least one antimicrobial resistance gene. The *aadA*, *streA*, *streB*, *erm* and *tetA* resistance genes were detected separately and/or concurrently. In conclusion, our findings indicate that environmental isolates of aEPEC O177 strains obtained from cattle in South Africa harboured virulence and antimicrobial resistance gene determinants similar to those reported in other shiga-toxin producing *E. coli* strains and suggest that these determinants may contribute to the virulence of the isolates.

***Keywords*:** Atypical enteropathogenic *E. coli* (aEPEC), bundle-forming pili (BFP), *E. coli* O177, virulence factors, antimicrobial resistance, shiga-toxins, diarrhoeagenic *E. coli*.

3.1. Introduction

Enteropathogenic *E. coli* (EPEC) is a group of diarrhoeagenic *E. coli* that is reported to cause high morbidity and mortality in humans, especially in immune-compromised subjects, elderly individuals and young children. EPEC are characterised by the presence of intimin (*eaeA*) genes coupled with the absence of the *stx* genes (Martins *et al*., 2016; Alonso *et al*., 2017). The *eaeA* gene is responsible for attaching and effacing (A/E) lesions on the intestinal epithelial cell of the host (Kaper *et al*., 2004; Martins *et al*., 2016; Malik *et al*., 2017). Based on the presence or absence of the EPEC adherence factor (EAF) plasmid, EPEC is subdivided into two groups that include typical Enteropathogenic *E. coli* (tEPEC) and atypical Enteropathogenic *E. coli* (aEPEC) (Trabulsi *et al*., 2002; Alonso *et al*., 2017). The tEPEC strains possess EAF plasmid, which encodes a bundle forming pili (*bfp*A) while aEPEC strains lack the *bfpA* gene (Canizalez-Roman *et al*., 2013; Malik *et al*., 2017). It is on these bases that

the virulence potentials of aEPEC is poorly understood and highly questioned. Despite the fact that tEPEC have been most often associated disease complications in humans, it is only recently that aEPEC been reported to cause diseases in both animals and humans (Malik *et al*., 2017). This may account for the reason why previous studies that have been documented worldwide and in the study area have focused on EHEC, especially *E. coli* O157 and non-O157 strains that received great attention due to its high pathogenicity (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011; Iwu *et al*., 2016; Jajarmi *et al*., 2017; Toro *et al*., 2018). The findings of most of these studies were in agreement with previous reports indicating that EHEC strains possessed *stx*$_1$, *stx*$_2$, *hlyA* and *eaeA* gene determinants that enhance their potential to cause infections such as diarrhoea, haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) in humans (Farrokh *et al*., 2013). However, to the best of our knowledge there is currently no report on the occurrence of EPEC, and aEPEC in particular among South African food-producing animals. This therefore creates a knowledge gap on the virulence profiles of aEPEC strains in the area. Despite the fact that aEPEC strains were known to be less pathogenic when compared to EHEC counterparts, data from some recent studies have reported the presence of virulence determinants in aEPEC strains thus making them to start receiving attention as pathogens of severe clinical significance in humans (Beutin *et al*., 2005; Bibbal *et al*., 2018) as well as in food-producing animals, especially sheep and wildlife (Otero *et al*., 2013; Álvarez-Suárez *et al*., 2016; Martins *et al*., 2016).

Even though extensive work has been done on shiga toxin producing *E. coli*, particularly O157 strain, no work has been conducted to investigate the virulence and antibiotic resistance profiles of aEPEC (*E. coli* O177) strains in food-producing animals, food, water and humans in South Africa. Hence, the aim of this study was to investigate the occurrence of aEPEC O177 serotype

from cattle and determine the presence of virulence and antibiotic resistance profiles of the isolates.

3.2. MATERIALS AND METHODS

3.2.1. Ethics statement

This study was approved by Animal Care Research Ethics Committee (AnimCare REC), North-West University-Mafikeng Campus, Ethical Clearance Certificate number: NWU-01223-19S9.

3.2.2. Samples collection

A total of 780 cattle faecal samples were collected from cattle from eight commercial farms (dairy and feedlot) within the North West province of South Africa. For confidentiality, the farms were given arbitrary names; CF-A, CF-B, CF-C, CF-D-D, CF-E, CF-F-D, CF-G and CF-H. The number of animals in these farms ranged from 120 to 2000 cattle head. Sampling was done between January 2017 and July 2017. The selection of the farms was based on the production system either intensive semi-intensive and/or extensive farming system in the study area and the wiliness of the farm owners to participate in the study. The letter requesting the permission to collect the samples was issued by the North West University and the permission was granted by both the farmers and the Department of Agriculture, Fisheries and Forestry (DAFF) in the study area. Ethical procedures such as restraining the animals using proper facilities and equipment were enforced during the collection of the samples. Animals showing any sign of diarrhoea or clinical signs were excluded. Faecal samples were collected directly from the rectum of individual animals using sterile arm-length gloves and in order to avoid duplication of sampling, the cattle were locked into their respective handling pens. Samples were placed in sterile sample collection bottles, labelled appropriately and immediately

transported on ice to the Molecular Microbiology laboratory, North-West University for microbial analysis.

3.2.3. Isolation of *E. coli* O177 strain

Approximately, 1 g of each sample was dissolved in 10% (w/v) saline solution (10 mL) and homogenized. Aliquots of 5 µL was transferred into 10 mL buffered peptone water. Ten-fold serial dilutions were prepared and aliquots of 100 µL from each dilution was spread-plated on Rainbow agarO157 plates (Kase *et al*., 2015). The plates were inverted and incubated aerobically at 37 °C for 24 hours. The colonies with dark pink or purple colour were randomly selected and purified by streaking on sorbitol MacConkey agar (Mast Group Ltd, Mayerside, UK) supplemented with 1 mg/L potassium tellurite and the plates were at 37 °C for 24 hours. Two colonies presenting pink colour were randomly picked and were used to prepare glycerol stocks and stored at −80 °C for future use.

3.2.4. Genomic DNA extraction from presumptive isolates

The glycerol stocks were resuscitated by streaking on MacConkey agar plates (Merck, SA) and incubated at 37°C for 24 hours. DNA extraction was performed using ZR Genomic DNA™– Tissue MiniPrep Kit (Zymo Research Corp, Irvine, USA) following the manufacturer's instructions. The concentration and purity of the DNA was determined using the NanoDrop Lite 1000 spectrophotometer -Thermo Fisher Scientific, USA. The extracted DNA was stored at −80 °C for PCR analysis.

3.2.5. Designing specific primers for detection of *E. coli* O177 strain

Serotype specific primer-pairs for detection of *E. coli* O177 strain were designed based on *rmlB* and *wzy* (encoding for dTDP-glucose 4, 6-dehydratase and polymerase, respectively) gene

fragments using the Primer3 software (Ye *et al*., 2012). Firstly, sequence data was retrieved from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) database and *Escherichia coli* serogroup O177 O-antigen gene cluster nucleotide sequences was obtained through DQ008593.1 accession number. The accession number (DQ008593.1) or FASTA sequence data was deposited into the NCBI and the primers were retrieved based on the selected genes (*rmlB* and *wzy*) from the template. Ten different primer pairs were generated per gene and the primers were selected based on physical parameters: C+G content, melting temperature, self-complimentary, secondary structure, product length and cross reactivity using Oligo version 7.6 4. Furthermore, selected primers were empirically subjected to Gene infinity platform to check for their potential to form prime-dimers, hairpin structure and for binding affinities to the priming sites. The specificity of the designed primers was empirically tested using NCBI/Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). An online bioinformatics tool, *in silico* PCR amplification was carried out to further determine the specificities of the primers (http://www.in silico.ehu.es/PCR/). Selected primers were synthesised by Inqaba Biotechnical Industry Ltd, Pretoria, South Africa.

3.2.6. Identification of aEPEC *E. coli* O177 strain using multiplex PCR assay

A singleplex PCR assay was performed for identification of the isolates, targeting *uidA* genus specific gene using previously described protocol (Anbazhagan *et al*., 2011). The developed multiplex PCR assay was empirically validated for its specificity, sensitivity, reproducibility and robustness. The designed primers were used for multiplex PCR assay to amplify *rmlB* and *wzy* gene fragments for detection of *E. coli* O177 strain. The primer sequences, targeted genes and amplicon sizes are listed in Table 2.1. The oligonucleotides were synthesised by Inqaba Biotechnical Industry Ltd, Pretoria, South Africa. The PCR reactions constituted 12.5 µL of 2X DreamTaq Green Master Mix, 0.5 µM (equimolar primer concentration of 10 µM) primer,

1 µg/µL of template DNA. The volume was adjusted to 25 µL with 10 µL RNase free PCR water. A non-DNA template (nuclease-free water) reaction tube served as a negative control while a DNA sample from *E. coli* O177 (isolated during the preliminary study) was used as positive control. All the PCR reagents used were Fermentas USA products supplied by Inqaba Biotec Industry Ltd, Sunnyside Pretoria, South Africa. Amplifications were performed using DNA thermal cycler (model- Bio-Rad C1000 Touch^{TM} Thermal Cycler). PCR conditions for amplification of *uidA*, *rmlB* and *wzy* gene fragments are shown in Table 3.1. All the PCR products were kept at 4 °C until gel electrophoresis was performed.

3.2.7. Detection of virulence genes in *E. coli* O177 isolates

The isolates were screened for the presence of *bfp* gene encoding for the bundle forming pili protein. *E. coli* O157 (*bfp*$^{+}$) (kindly supplied by Prof Ateba, Department of Molecular Microbiology, North-West University, South Africa) was used as positive control. All *E. coli* O177 isolates which were negative for *bfp* gene were classified as aEPEC strains. The isolates were further screened for the presence of medically important virulence genes that included *stx*$_1$, *stx*$_2$, *eaeA, hlyA* and *stx*$_2$ variants (*stx*$_{2a}$, *stx*$_{2b}$, *stx*$_{2c}$, *stx*$_{2d}$, *stx*$_{2e}$, *stx*$_{2f}$ and *stx*$_{2g}$) using PCR protocols as previously described (Paton and Paton, 1998, He *et al*., 2012). The primer sequences, targeted genes and amplicon sizes are listed in Table 3.1. PCR reactions were prepared in 25 µL volumes and amplifications were performed using DNA thermal cycler (Model- Bio-Rad C1000 Touch^{TM} Thermal Cycler). For quality control, DNA extracted from *E. coli* O157:H7 (environmental strain isolated sequenced from our research group) and *E. coli* ATCC 98222 (non-pathogenic strain) were used as positive (*bfp*$^{+}$, *stx1*, *stx*$_2$, *eaeA,* and *hlyA* genes) and negative controls, respectively. All the PCR products were kept at 4 °C.

3.2.8. Antimicrobial susceptibility test

The antimicrobial susceptibility phenotype of the isolates was determined using a Kirby-Bauer disk diffusion method (Bauer *et al*., 1966), following the standard and interpretive criteria outlined by Clinical and Laboratory Standards Institute (CLSI, 2016). A 10 mL of overnight culture for each isolate was prepared in a 15 mL falcon tubes using sterile nutrient broth and incubated at 37 °C for 24 hours. The turbidity of the cultures was adjusted to 1 x 10^8 CFU/mL (approximately equivalent to 0.5 McFarlan standard) using Thermo Spectronic (Model, Helios Epsilon). Aliquot of 50 µL of the culture was spread on Mueller Hinton agar plates. The following antibiotics discs and concentrations were placed on inoculated plates: Ampicillin (AMP), 10 µg; Chloramphenicol (C), 30 µg; Erythromycin (E), 15 µg; Kanamycin (K), 30 µg; Norfloxacin (NOR), 10 µg; Streptomycin (S), 10 µg and Tetracycline (TE), 30 µg. The discs were supplied by Davis Diagnostic Laboratory (pty Ltd, South Africa). The plates were incubated at 37 °C for 24 hours. The results were interpreted and recorded based on the CLSI guidelines (CLSI, 2016). *E. coli* ATCC 25922 was used as a reference for internal control.

3.2.9. Detection of genetic determinants for antibiotic resistance genes by PCR

The *E. coli* O177 isolates, which were resistant to at least three categories of antimicrobial agents tested were screened for the presence of antibiotic resistance determinants viz; *tetA*, *tetW, aadA*, *strA*, *strB*, *ampC*, *cmlA*, *ermB* and *kan* genes. The primer sequences, targeted resistance genes and amplicon sizes are listed in Table 3.2. The reactions were prepared in 25 µL volumes and amplifications were performed using DNA thermal cycler (Model- Bio-Rad C1000 Touch™ Thermal. Amplicons were store at 4 °C until the gel electrophoresis was performed.

3.2.10. Agarose gel electrophoresis

All PCR amplicons were resolved by electrophoresis on a 2% (w/v) agarose gel containing 0.001 µg/mL ethidium bromide. A 100 bp DNA molecular weight DNA marker (Fermentas, USA) was included in each gel and used to confirm the sizes of the amplicons. Electrophoresis was conducted in a horizontal Pharmacia biotech equipment system (Model Hoefer HE 99X, Amersham Pharmacia biotech, Sweden) for 1 hour at 80V using 1X TAE buffer. A ChemiDoc Imaging System (Bio-RAD ChemiDoc^{TM} MP Imaging System, UK) was used to capture the image using Gene Snap (version 6.00.22) software.

3.2.11. Nucleotide sequences analysis

Amplified *rmlB* and *wzy* gene fragments were sequenced by Inqaba Biotec, Pretoria-South Africa. Nucleotide sequence data was cleaned using the ChromasLite software version 2.6.5. In addition, BioEdit Sequence Alignment Editor was used to align the sequences (sense and antisense) and the consensus sequence was created. Then the consensus sequences obtained were deposited in the NCBI using Blast web-tool (https://www.ncbi.nlm.nih.gov/Blast) to determine the most probable similarities of the organism in question; *E. coli* O177 (Altschul *et al*., 1997). In addition, nucleotide sequence data (Seg1, Seg2 and Seq3) was submitted in the GenBank using Bankit submission platform (http://www.ncbi.nlm.nih.gov/) to obtain the accession number for the sequences.

Table 3.1: Oligonucleotide primers used for amplification of the various targeted virulence genes in *E. coli* O177 strain.

Primers	Sequence	Target genes	Amplicon size (bp)	PCR conditions	References
I-Identification					
UidA-F	CTGGTATCAGCGCGAAGTCT	*uidA*	600	95 °C, 10 min(1x); 95 °C, 45 sec; 59 °C, 30 sec; 72 °C, 90 sec (35x); 72 °C for 10 min (1x)	Anbazhagan *et al*. (*2011*)
UidA-R	AGCGGGTAGATATCACACTC				
RmlB-F	CGCGGATTTTTGCTCTGCAT	*rmlB*	645	95 °C,3 min (1x); 94 °C, 30 sec; 55 °C, 30 sec; 72°C, 60 sec (30x); 72 °C for 5 min (1x)	This study
RmlB-R	CAGTAATTGCGAGCCGCTTC				
Wzy-F	GGTCAGGAGCATGGAGCATT	*wzy*	457		
Wzy-R	AATCCATCCGGTGTATCGGC				
II-Virulence genes					
Bfp-F	AATGGTGCTTGCGCTTGCTGC	*bfp*	324	95 °C,3 min (1x); 94 °C, 60 sec; 64 °C, 60sec; 72 °C, 2 min (35x); 72 °C for 10 min (1x)	Ghanbarpour *et al*. (2017)
Bfp-R	GCC GCTTTATCCAACCTGGTA				
EaeA-F	GACCCGGCACAAGCATAAGC	*eaeA*	384	95 °C, 10 min (1x); 95 °C, 60 sec; 62 °C, 2 min; 72 °C, 90 sec (35x), 72 °C, 5 min (1x)	
EaeA-R	CCACCTGCAGCAACAAGAGG				
HlyA-F	GCATCATCAAGCGTACGTTCC	*hlyA*	534		

HlyA-R	AATGAGCCAAGCTGGTTAAGCT			95 °C, 10 min (1x); 95 °C, 60 sec; 64 °C, 2 min; 72°C, 90 sec (35x), 72 °C, 5 min (1x)	Paton and Paton (1998)
Stx1-F	ATAAATCGCCATTCGTTGACTAC	*stx*$_{1}$	180	95 °C, 10 min (1x); 95 °C, 60 sec; 62 °C, 2 min; 72 °C, 90 sec (35x), 72 °C, 5 min (1x)	
Stx1-R	AGAACGCCCACTGAGATCATC				
Stx2-F	GGCACTGTCTGAAACTGCTCC	*stx*$_{2}$	255		
Stx2-R	TCGCCAGTTATCTGACATTCT				
Stx2a-F	AGATATCGACCCCTCTTGAA	*stx*$_{2a}$	969	94 °C, 5 min (1x); 94 °C, 45 sec; 60 °C, 45 sec; 72 °C, 90 sec (25x); 72 °C, 7 min (1x)	He *et al*. (2012)
Stx2a-R	GTCAACCTTCACTGTAAATG				
Stx2-G2-F	TATACGATGACACCGGAAGAAG	*stx*$_{2b}$	300	94 °C, 5 min (1x); 94 °C, 45 sec; 65 °C, 30 sec; 72 °C, 60 sec (25x); 72 °C for 5 min (1x)	
Stx2-G2-R	CCTGCGATTCAGAAAAGCAGC				
Stx2/2c	TTTTATATACAACGGGTA	*stx*$_{2c}$	163	94 °C, 5 min (1x); 94 °C, 45 sec; 51 °C, 30 sec; 72 °C, 60 sec (30x); 72 °C, 5 min (1x)	
Stx2-G1-R	GGCCACTTTTACTGTGAATGTA				
Stx2d-act	CTTTATATACAACGGGTG	*stx*$_{2d}$	359	94 °C, 5 min (1x); 94 °C, 60 sec; 50°C, 60 sec; 72 °C, 60 sec(25x); 72 °C, 5 min (1x)	
CKS2	CTGAATTGTGACACAGATTAC				
Stx2-G4-F	CAGGAAGTTATATTTCCGTAGG	*stx*$_{2e}$	911	94 °C, 5 min (1x); 94 °C, 30 sec; 55 °C, 30 sec; 72 °C, 60 sec (25x); 72 °C, 5 min (1x)	
Stx2-G4-R	GTATTCTCTTCCTGACACCTTC				

Stx2-G3-F	TTTACTGTGGATTTCTCTTCGC	*stx*$_{2f}$	875	94 °C, 5 min (1x); 94 °C, 30 sec; 61 °C, 30 sec; 72 °C, 60 sec (25x); 72 °C for 5 min (1x)	
Stx2-G3-R	TCAGTAAGATCCTGAGGCTTG				
209-F	GTTATATTTCTGTGGATATC	*stx*$_{2g}$	573	94 °C, 5 min (1x); 94 °C, 45 sec; 55 °C, 60 sec; 72 °C, 60 sec (25x); 72 °C for 7 min (1x)	
781-R	GAATAACCGCTACAGTA				

Table 3.2: Oligonucleotide primers used for amplification of the various antibiotic resistance genes in *E. coli* O177 strain.

Primers	**Sequence**	**Target genes**	**Amplicon size (bp)**	**PCR conditions**	**References**
TetA-F	GCTACATCCTGCTTGCCTTC	*tet(A)*	210	95 °C, 3 min (1x); 94 °C, 30 sec; 58 °C, 30 sec; 72 °C, 60 sec (35x); 72 °C, 5 min (1x)	Bergeron *et al*. (2015)
TetA-R	CATAGATCGCCGTGAAGAGG				
TetW-F	GAGAGCCTGCTATATGCC AGC	*tet(W)*	168		
TetW-R	GGGCGTATCCACAATGTTAAC				
ErmB-F	GATACCGTTTACGAAATTGG	*ermB*	364	95 °C, 60 sec; 94 °C, 15 sec; 56 °C, 30 sec; 72 °C, 60 sec (40x) 72 °C, 5 min (1x)	
ErmB-R	GAATCGAGACTTGAGTGTGC				

AadA-F	GTGGATGGCGGCCTGAAGCC	*aadA*	525	95 °C, 3 min (1x); 94 °C, 30 sec; 55 °C, 30 sec; 72 °C, 60 sec (35x), 72 °C, 5 min (1x)	Srinivasan *et al*. (2007)
AadA-R	AATGCCCAGTCGGCAGCG				
StrA-F	CCTGGTGATAACGGCAATTC	*strA*	549		
StrA-R	CCAATCGCAGATAGAAGGC				
StrB-F	ATCGTCAAGGGATTGAAACC	*strB*	509		
StrB-R	GGATCGTAGAACATATTGGC				
CmlA-F	CCGCCACGGTGTTGTTGTTATC	*cmlA*	698		
CmlA-R	CACCTTGCCTGCCCATCATTAG				
AmpC-F	CATATGCTTAATCAGTGAGGCACCT	*ampC*	850	95 °C, 3 min (1x); 95 °C, 60 sec; 59 °C, 60 sec; 72 °C, 2 min (35x), 72 °C, 10 min (1x)	Samra *et al*. (2009)
AmpC-R	GAATTCAGTATTCAACATTTCCGTGTCG				
Kan-F	CATATGAGAAAAACTCATCGAGCATC	*kan*	810		
Kan-R	GAATTCAGCCATATTCAACGGGAA				

3.2.12. Statistical analysis

Measured parameters were tested for normality using the NORMAL option in the Proc Univariate statement as well as for homogeneity of variances using the Levene's test option in the Means statement of the GLM procedure of SAS prior to analysis of variance. Data on the proportions of samples positive for *E. coli* O177 strain were analysed for effect of farming system using the general linear models (GLM) procedures of SAS (2010) according to the following statistical model:

$$Y_{ij} = \mu + F_i + E_{ij}$$

where, Y_{ij} = response variable, μ = overall mean, F_i = farming system (intensive, semi-intensive and extensive) effect and E_{ij} = random error associated with observation *ij*, assumed to be normally and independently distributed. Data on number of isolates carrying virulent genes, number of antibiotic resistant isolates, number of isolates carrying antibiotic resistance genes, and number of multi-drug resistant isolates were square root-transformed before statistical analysis using the GLM procedures (SAS, 2010). For all statistical tests, significance was declared at $P < 0.05$.

3.3. Results

3.3.1. Identification of *E. coli* O177 strain using multiplex PCR analysis

A total of 780 cattle faecal samples were analysed for the presence of *E. coli* O177 using Rainbow agarO157 and a total of 1272 non-repetitive presumptive isolates were obtained. A 915 of the isolates were successfully identified as *E. coli* isolates through amplification of the *uid*A genus-specific gene, Figure 3.1. All the 915 *E. coli* isolates were further screened for the presence of *E. coli* O177 strain by amplifying *rmlB* and *wzy* genes using multiplex PCR

analysis. Out of 915 isolates screened, 376 were identified as *E. coli* O177 strain, Table 3.3. Figure 3.2 indicates a 2% (w/v) agarose gel of representative *rmlB* and *wzy* gene fragments amplified in the study. The occurrence of *E. coli* O177 strain was 33.3 ± 14.43%, 33.3 ± 14.43%, and 50.0 ± 14.18% in intensive, semi-intensive and extensive farming systems, respectively. However, no significant ($p > 0.05$) differences were observed on the occurrence of *E. coli* O177 strain across intensive, semi-intensive and extensive animal production systems.

Table 3.3: Results of isolation and identification of *E. coli* O177 from commercial farms in the North-West province, South Africa.

Farms	**No. of samples collected per farm**	**Presumptive isolates**	**Confirmed *E. coli* isolates**	**Confirmed *E. coli* O177 isolates**
CF-A	100	114	102	70
CF-B	80	100	75	23
CF-C	80	130	86	44
CF-D-D	120	230	173	91
CF-E	80	140	92	25
CF-F-D	80	130	115	40
CF-G	80	178	87	34
CF-H	160	250	185	49
Total	**780**	**1272**	**915**	**376**

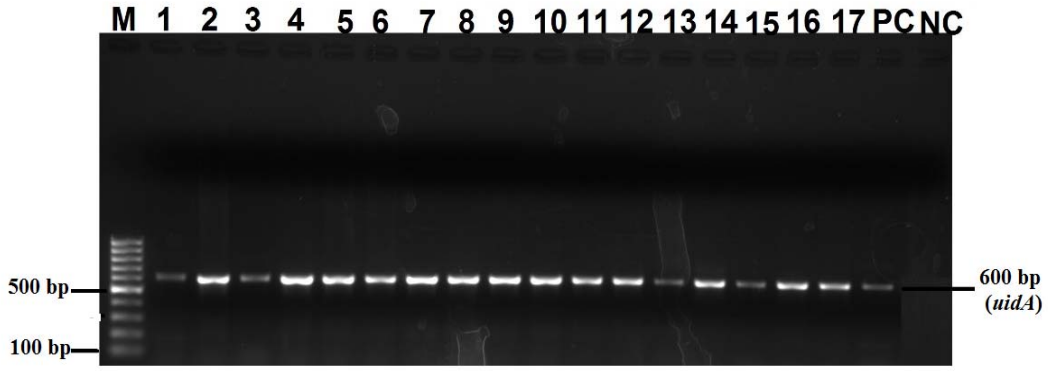

Figure 3.1: Agarose gel [2%] image depicting *uidA* gene fragments amplified from DNA samples extracted from *E. coli* isolates. Lane M = 100 bp molecular marker; Lane 1-17 = *uidA* (**600 bp**) gene fragments amplified from *E. coli* isolates, Lane PC = *uidA* gene fragment amplified from *E. coli* strain and lanes NC = Negative control.

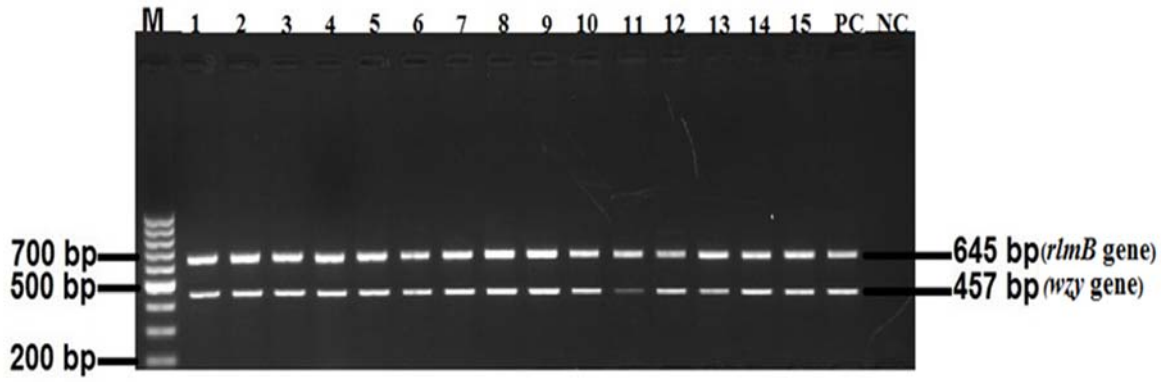

Figure 3.2: Agarose gel [2%] image depicting *rmlB* and *wzy* gene fragments amplified from *E. coli* O177 isolates. Lane M = 100 bp molecular marker; Lane NC = Negative control (without DNA template); Lane PC = *rmlB* and *wzy* gene fragments amplified from *E. coli* O177 isolates; Lanes 1-15 = *rmlB* and *wzy* (**645** and **457 bp,** respectively) gene fragments amplified from *E. coli* O177 isolates.

3.3.2. Detection of virulence genotypes in *E. coli* O177 isolates

A total of 376 atypical *E. coli* O177 isolates (confirmed by PCR) were screened for the presence of four main virulence genes; *bfpA*, *eaeA*, *hlyA*, *stx*$_1$, *stx*$_2$ and *stx*$_2$ subtypes, using PCR analysis. The PCR results revealed that none of the isolates possessed *bfpA* gene fragment and thus were classified as aEPEC. Nevertheless, *eaeA*, *hlyA*, *stx*$_1$, *stx*$_2$, *stx*$_{2a}$ and *stx*$_{2d}$ subtypes were detected

in 350 isolates. Generally, 350 isolates harboured these virulence genes. Figures 3.4-3.8 show gene fragments (*hlyA*, *stx*$_2$, *stx*$_1$, *eaeA*, *stx*$_{2a}$ and *stx*$_{2d}$ subtypes, respectively) detected in *E. coli* O177 isolates. Significant differences ($p < 0.001$) in terms of occurrence of various virulence genes in *E. coli* O177 isolates was observed. The *hlyA* (12.74%) and *stx*$_2$ (11.20%) were the most commonly detected genes ($p < 0.001$), followed by *stx*$_1$ (9.07%) and *eaeA* (7.25%), Figure 3.9. Two *stx*$_2$ subtypes namely; the *stx*$_{2a}$ and *stx*$_{2d}$, were detected among the isolates. A few of the isolates (2.60%) harbored *stx*$_{2a}$, while 0.63% possessed *stx*$_{2d}$ gene. The majority (9.74%) of the isolates carried a combination of *hlyA/stx*$_2$ while 7.40%, 7.09%, 6.80% and 5.55% possessed *stx*$_1$/*stx*$_2$, *stx*$_1$/*stx*$_2$/*hlyA*, *hlyA/eaeA* and *stx*$_2$/*hlyA/eaeA*, respectively ($p < 0.001$). There was no difference ($p > 0.05$) between the occurrences of *stx*$_1$/*eaeA* (6.35%) and *stx*$_1$/*stx*$_2$/*hlyA*/*eaeA* (5.11%) in *E. coli* O177 isolates. Only one isolate possessed *stx*$_1$/*stx*$_2$/*stx*$_{2a}$/*stx*$_{2d}$/*hlyA*/*eaeA*. Despite the fact that none of the isolates possessed the *stx*$_2$ subtypes *stx*$_{2b}$, *stx*$_{2c}$, *stx*$_{2e}$, *stx*$_{2f}$ and *stx*$_{2g}$, the virulence gene profiles of these aEPEC strains especially the isolate with the *stx*$_2$ subtypes was a cause for concern.

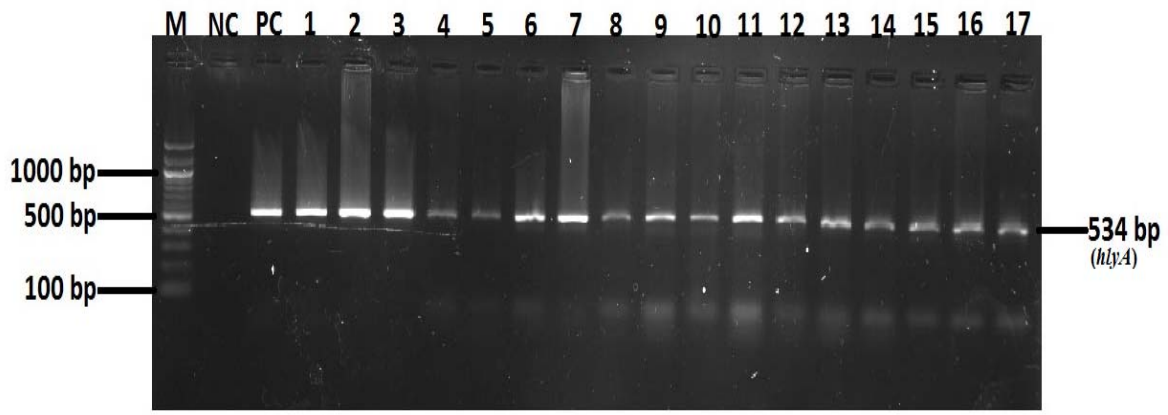

Figure 3.3: Agarose gel [2%] image depicting *hlyA* gene fragments amplified from DNA samples extracted from *E. coli* O177 isolates. Lane M = 100 bp molecular marker; Lane NC = Negative control (DNA template from *E. coli* ATCC 98222); Lane PC = *hlyA* gene fragments amplified from *E. coli* O157:H7 strain; Lanes 1-17 = *hlyA* (**534 bp**) gene fragments amplified from *E. coli* O177 isolates.

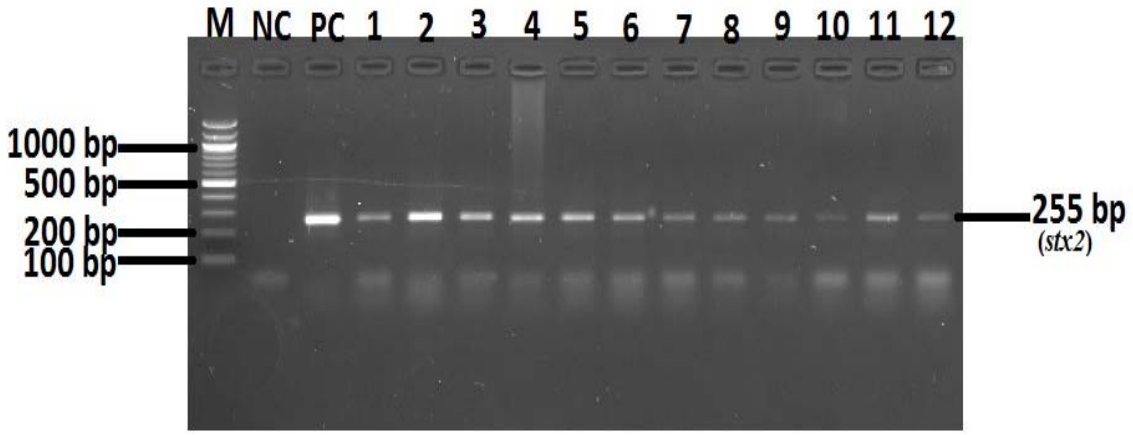

Figure 3.4: Agarose gel [2%] image depicting *stx*$_2$ gene fragments amplified from DNA samples extracted from *E. coli* O177 isolates. Lane M = 100 bp molecular weight marker; Lane NC = Negative control (DNA template from *E. coli* ATCC 98222); Lane PC = *stx*$_2$ gene fragments amplified from *E. coli* O157:H7 strain; Lanes 1-12 = *stx*$_2$ (**255 bp**) gene fragments amplified from *E. coli* O177 isolates.

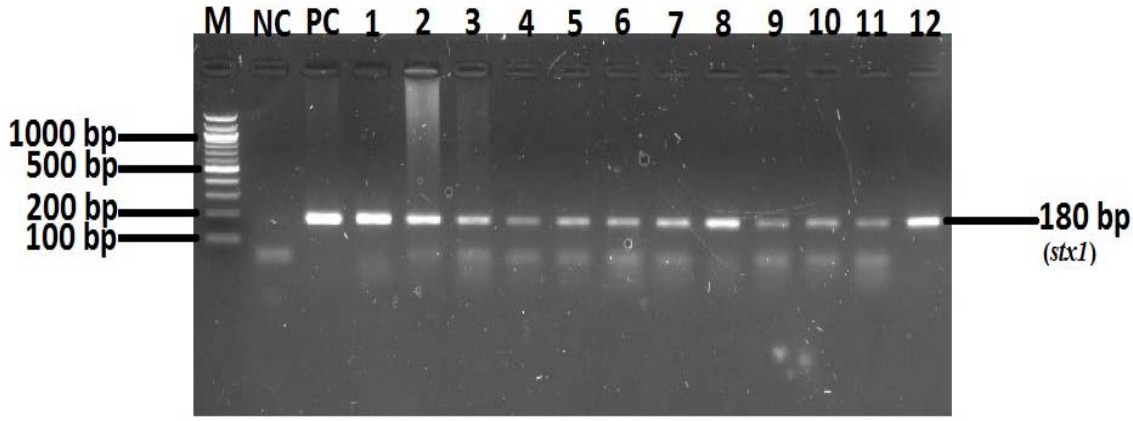

Figure 3.5: Agarose gel [2%] image depicting *stx*$_1$ gene fragments amplified from DNA samples extracted from *E. coli* O177 isolates. Lane M = 100 bp molecular marker; Lane NC = Negative control (DNA template from *E. coli* ATCC 98222); Lane PC = *stx*$_1$ gene fragments amplified from *E. coli* O157:H7 strain; Lanes 1-12 = *stx*$_1$ (**180 bp**) gene fragments amplified from *E. coli* O177 isolates.

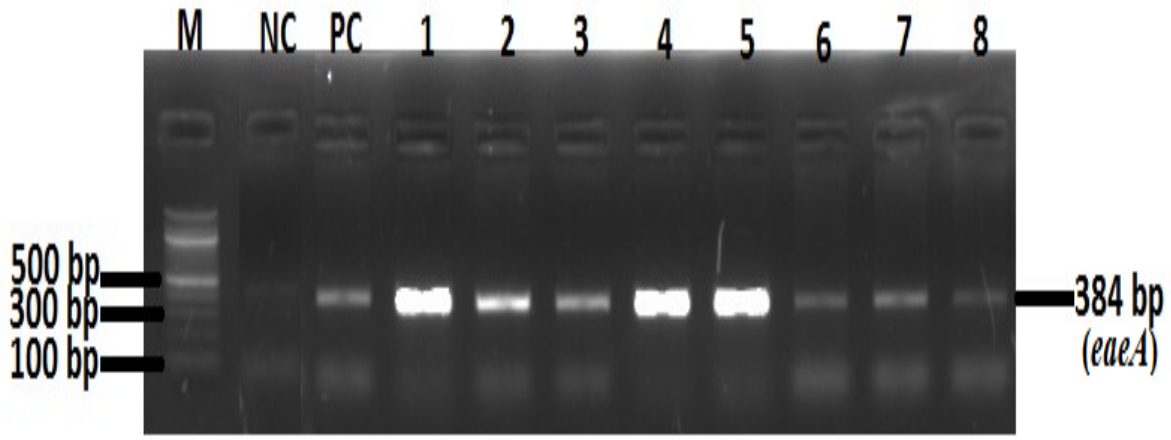

Figure 3.6: Agarose gel [2%] image depicting *eaeA* gene fragments amplified from DNA samples extracted from *E. coli* O177 isolates. Lane M = 100 bp molecular marker; Lane NC = Negative control (DNA template from *E. coli* ATCC 98222); Lane PC = *eaeA* gene fragments amplified from *E. coli* O157:H7 strain; Lanes 1-18 = *eaeA* (**384 bp**) gene fragments amplified from *E. coli* O177 isolates.

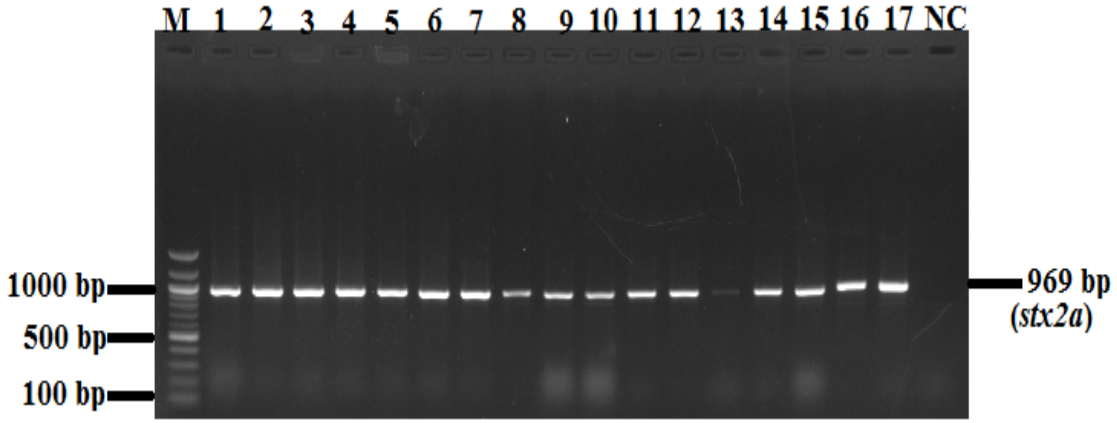

Figure 3.7: Agarose gel [2%] image depicting *stx*$_{2a}$ gene fragments amplified from DNA samples extracted from *E. coli* O177 isolates. Lane M = 100 bp molecular weight marker; Lane NC = Negative control (DNA template from *E. coli* ATCC 98222); Lanes 1-17 = *stx*$_{2a}$ (**969 bp**) gene fragments amplified from *E. coli* O177 isolates.

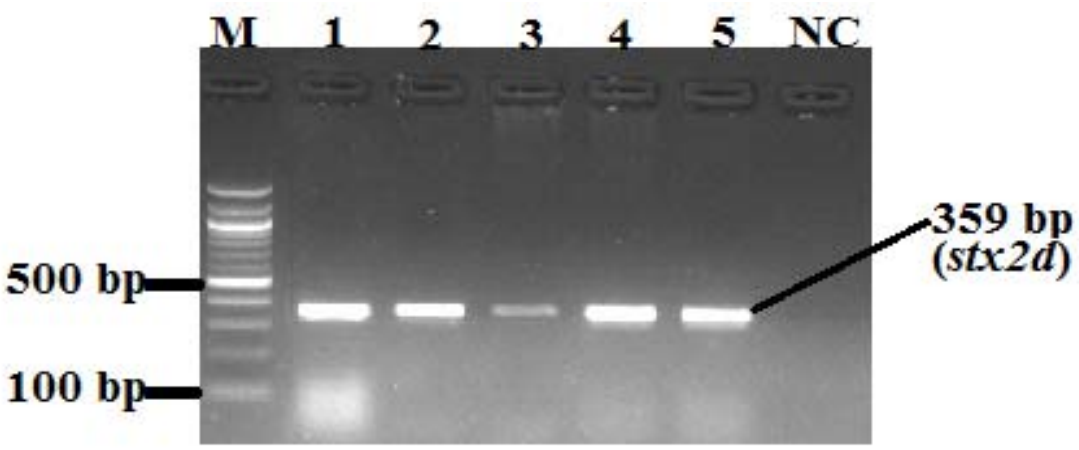

Figure 3.8: Agarose gel [2%] image depicting *stx*$_{2d}$ gene fragments amplified from DNA samples extracted from *E. coli* O177 isolates. Lane M = 100 bp molecular weight marker; Lane NC = Negative control (DNA template from *E. coli* ATCC 98222); Lanes 1-5 = *stx*$_{2d}$ (**359 bp**) gene fragments amplified from *E. coli* O177 isolates.

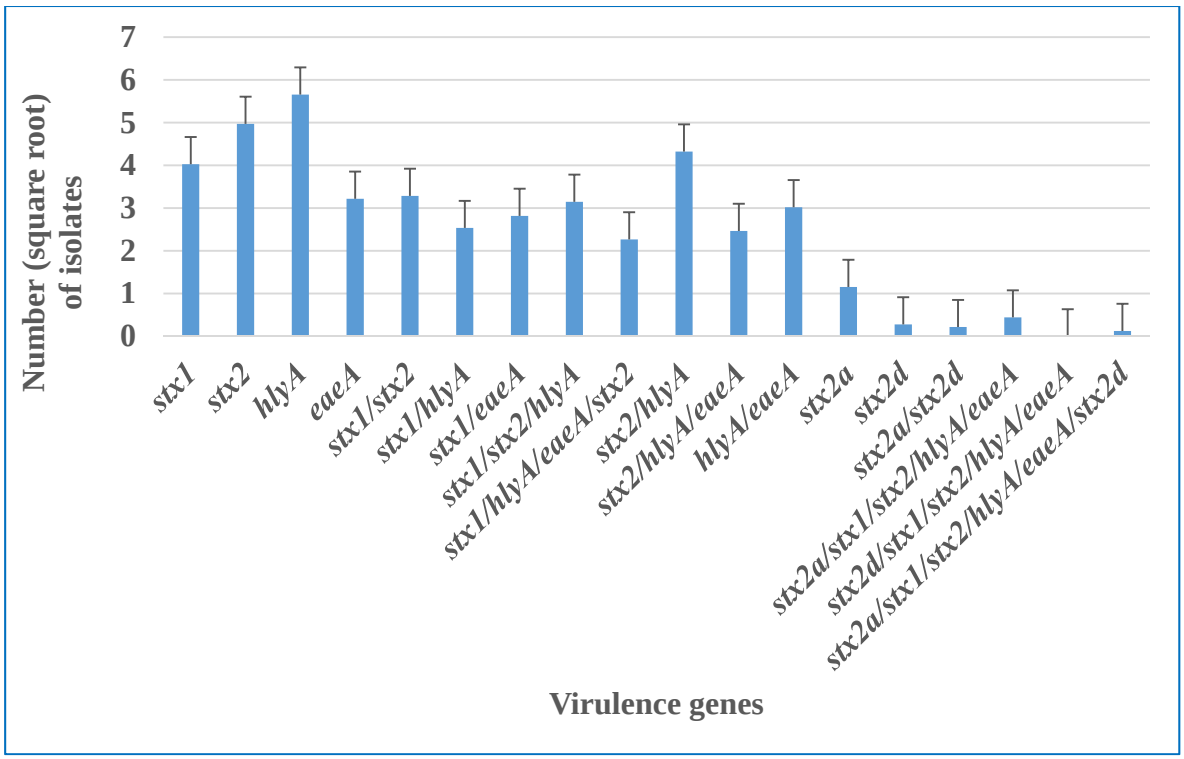

Figure 3.9: Distribution of virulence genes in *E. coli* O177 strain isolated from cattle faeces. The bars indicate the standard error ($P < 0.05$).

3.3.3. Antimicrobial resistance profiles

Antimicrobial susceptibility tests of the 376 *E. coli* O177 isolates revealed diverse antimicrobial resistance pattern against all the seven antibiotics tested. The isolates revealed various resistance patterns against different antibiotics. A large proportion (63.84%) *E. coli* O177 isolates were resistant to erythromycin compared to other antibiotics ($p < 0.001$), Figure 3.10. No difference significant ($p > 0.05$) was observed in *E. coli* O177 resistance to ampicillin (21.54%), tetracycline (13.37%), streptomycin (17.01%), kanamycin (2.42%), chloramphenicol (1.97%) and norfloxacin (1.40%). Resistance to at least two antibiotics tested was observed. Most of the isolates (20.74%) exhibited high level of resistance to three or more antibiotics, Figure 3.11 ($p < 0.001$). The MAR index ranged from 0.29 to 0.86 whereas average MAR index was 0.65.

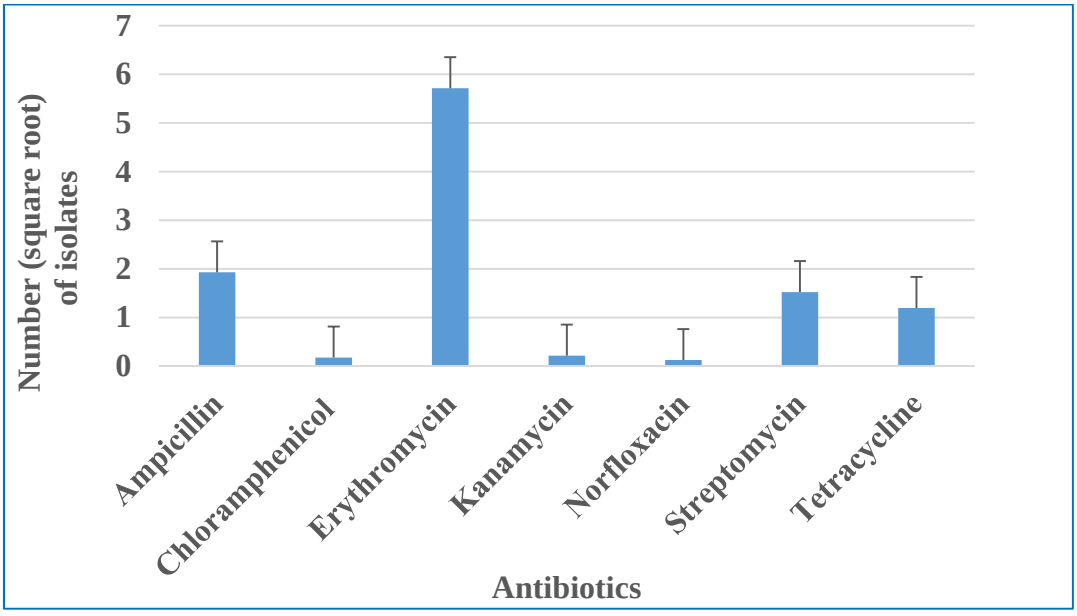

Figure 3.10: Antibiotic resistance pattern of *E. coli* O177 strain isolated from cattle faeces. The bars indicate the standard error ($P < 0.05$).

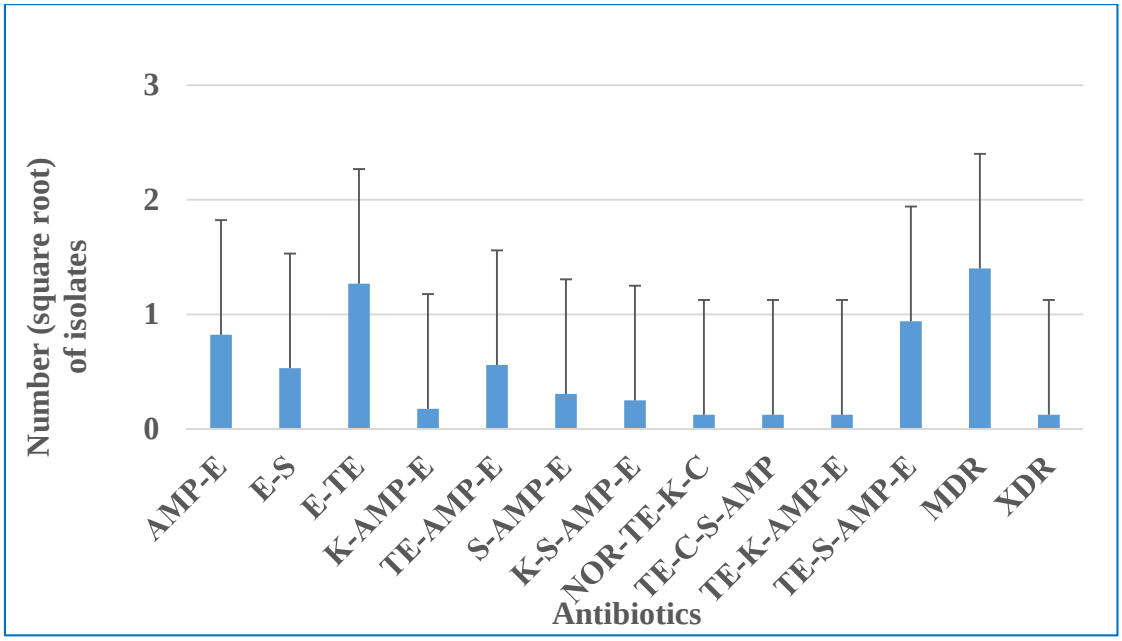

Figure 3.11: Multiple resistance patterns of *E. coli* O177 isolates obtained from cattle faeces. ***Key*:** AMP = ampicillin; C = chloramphenicol; E = erythromycin; K = kanamycin; NOR = norfloxacin; TE = tetracycline; S = streptomycin; MDR = multi-drug resistant; XDR = extensively drug-resistant. The bars indicate the standard error ($P < 0.05$).

3.3.4. Detection of antimicrobial resistance genes

Antimicrobial resistance determinants were detected in all (73) the *E. coli* O177 isolates screened. Although there were ten genes investigated, only five gene fragments were successfully amplified, Figure 3.12 - 3.16. The most prevalent antimicrobial resistance genes detected are known to confer resistance to streptomycin and erythromycin, Figure 3.17. Genes *aadA* (22.86%), *strA* (19.85%), *strB* (19.38%), *ermB* (19.68%) and *tetA* (18.23%) were frequently detected. However, there was no significant ($p > 0.05$) means differences in the occurrence of these genes in *E. coli* O177 isolates. No isolates possessed the *tetW* gene. Some isolates harboured a combination of three or more antibiotic resistance genes. Most (24%) of the isolates carried a combination of the *aadA*, *strA* and *strB* genes while a smaller proportion 11% isolates possessed all the five antimicrobial resistance genes (*aadA*/*strA*/*strB*/*ermB*/*tetA*) ($P < 0.05$). However, none of the genes associated with ampicillin, chloramphenicol,

kanamycin and norfloxacin (*ampC, cmlA* and *kan*, respectively) were detected in all the isolates screened.

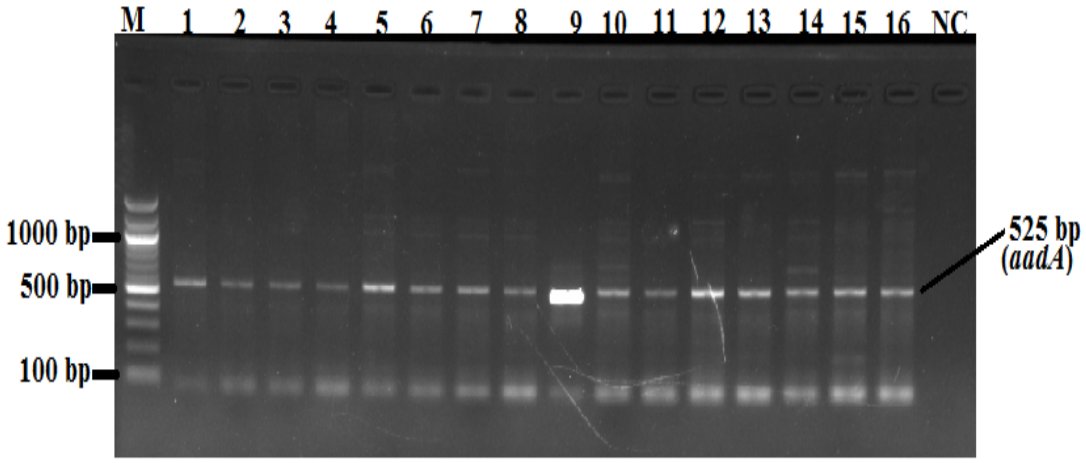

Figure 3.12: Agarose gel [2%] image depicting *aadA* gene fragments amplified from DNA samples extracted from *E. coli* O177 isolates. Lane M = 100 bp molecular weight marker; Lane NC = Negative control (without DNA template); Lanes 1-16 = *aadA* (**525 bp**) gene fragments amplified from *E. coli* O177 isolates.

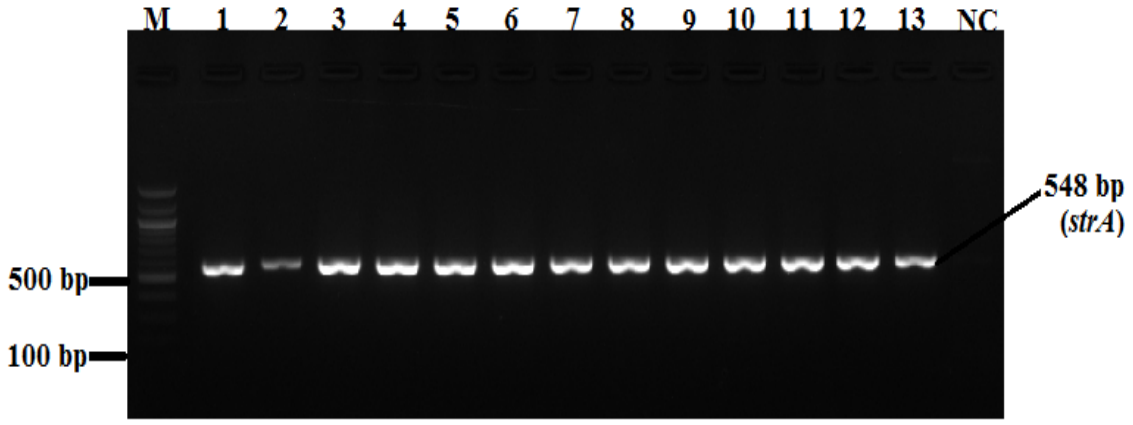

Figure 3.13: Agarose gel [2%] image depicting *strA* gene fragments amplified from DNA samples extracted from *E. coli* O177 isolates. Lane M = 100 bp molecular weight marker; Lane NC = Negative control (without DNA template); Lanes 1-13 = *strA* (**548 bp**) gene fragments amplified from *E. coli* O177 isolates.

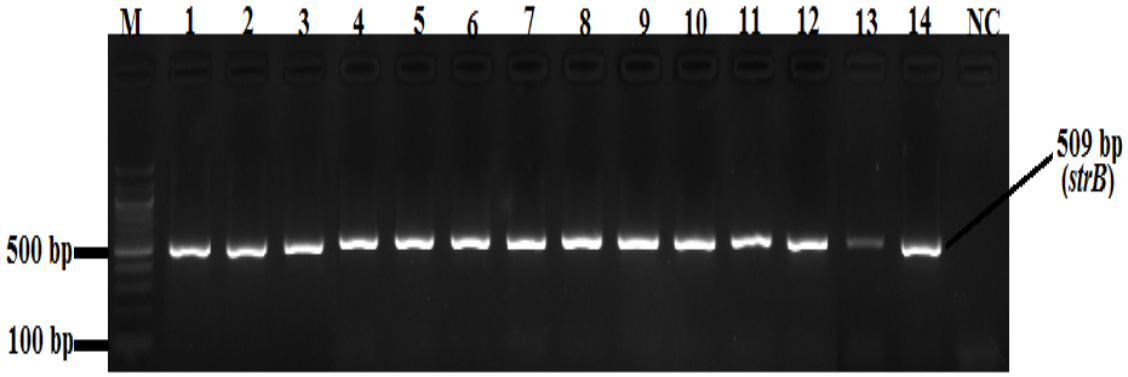

Figure 3.14: Agarose gel [2%] image depicting *strB* gene fragments amplified from DNA samples extracted from *E. coli* O177 isolates. Lane M = 100 bp molecular weight marker; Lane NC = Negative control (without DNA template); Lanes 1-14 = *strB* (**509 bp**) gene fragments amplified from *E. coli* O177 isolates.

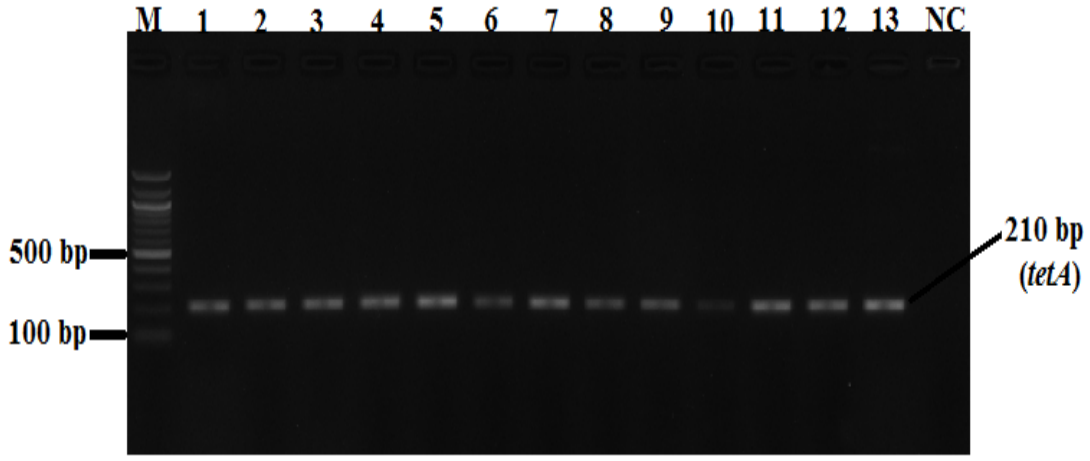

Figure 3.15: Agarose gel [2%] image depicting *tetA* gene fragments amplified from DNA samples extracted from *E. coli* O177 isolates. Lane M = 100 bp molecular weight marker; Lane NC = Negative control (without DNA template); Lanes 1-13 = *tetA* (**210 bp**) gene fragments amplified from *E. coli* O177 isolates.

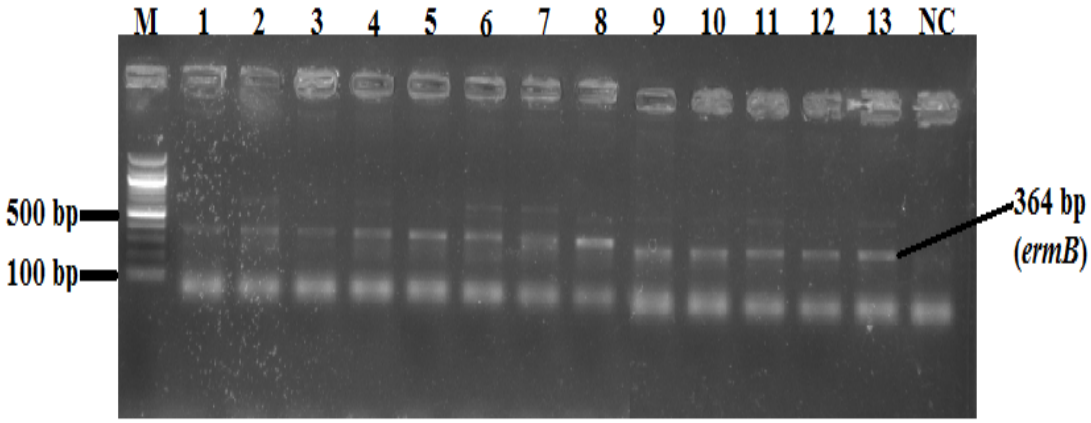

Figure 3.16: Agarose gel [2%] image depicting *ermB* gene fragments amplified from DNA samples extracted from *E. coli* O177 isolates. Lane M = 100 bp molecular weight marker; Lane NC = Negative control (without DNA template); Lanes 1-13 = *ermB* (**364 bp**) gene fragments amplified from *E. coli* O177 isolates.

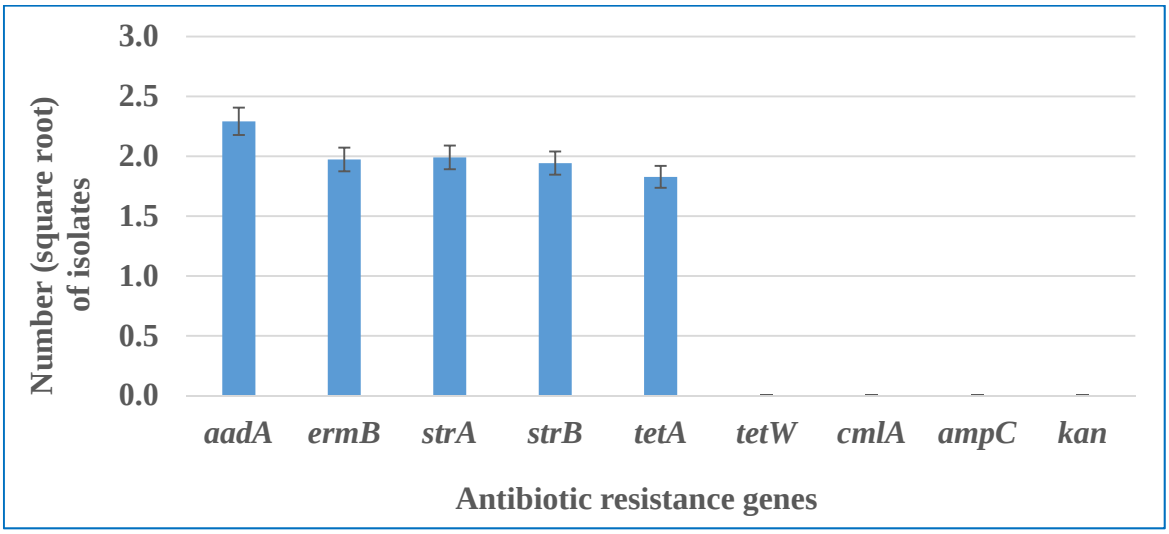

Figure 3.17: Distribution of virulence genes in *E. coli* O177 isolates obtained from cattle faeces. The bars indicate the standard error (±) ($P < 0.05$).

3.3.5. Sequence identifier and accession numbers

Sequence data analysis revealed 97% homology to *E. coli* O177 serogroup. The nucleotide sequences for Seq1, Seq2 and Seq3 were assigned GenBank accession numbers; MH389799, MH389800 and MH389801, respectively.

3.4. Discussion

In this study, the occurrence of aEPEC from cattle faeces, virulence genes and antibiotic resistance profiles of *E. coli* O177 isolates were analysed to determine their virulence potential. Although data on virulence and antibiotic resistance profiles of aEPEC strains from food producing animals is very rare, recent studies have reported virulence potential of these strains (Otero *et al*., 2013; Martins *et al*., 2016; Álvarez-Suárez *et al*., 2016; Ingle *et al*., 2016). Cattle are primary reservoir of *E. coli* species and contaminated meat and food products derived from cattle and other food-producing animals have been implicated in foodborne infections (Rios *et al*., 2019; Ateba and Mbewe, 2011). Despite the fact that few studies have reported the occurrence of aEPEC strains in food-producing animals, food products and farm environments (Horcajo *et al*., 2012; Otero *et al*., 2013; Álvarez-Suárez *et al*., 2016; Ghanbarpour *et al*., 2017; Rios *et al*., 2019), none of them have investigated the presence of both antibiotic resistance and virulence genes associated with STEC in atypical enteropathogenic *E. coli* O177 strain.

In this study, *E. coli* was successfully isolated from cattle faeces using Rainbow agarO157. The identities of the isolates were confirmed through amplification of *uidA* gene fragments (Anbazhagan *et al*., 2011). Moreover, multiplex PCR protocol was developed and targeted r*mlB* and *wzy* O-antigen gene cluster to identify *E. coli* O177 strain among the isolates. This protocol revealed high specificity and reproducibility for the detection of *E. coli* O177 strain from cattle faeces. And based on its robustness and efficacy in detecting *E. coli* O177, this protocol could be used to detect and discriminate *E. coli* O177 from other serotypes isolated from food, water and other environmental samples. Furthermore, the occurrence of *E. coli* O177 in cattle reported in this study is higher (41.09%) than those reported in the previous studies 19.2%, (Martins *et al*., 2016), 4.5% (Ghanbarpour *et al*., 2017) and 9.0% (Haymaker *et*

al., 2019). This disparity could be attributed to different sample sources, geographical location and the sensitivity of the methods used.

Given that aEPEC strains lack medically important virulence factors, its ability to cause diseases particularly in humans remains unclear (Otero *et al*., 2013; Ingle *et al*., 2016). Although aEPEC strains are characterized by lack of *stx*, *bfpA* genes, few studies have established that aEPEC may harbour virulence genes similar to those found in STEC strains (Horcajo *et al*., 2012; Haymaker *et al*., 2019; Rios *et al*., 2019). Against this backdrop, *bfpA* and other primary virulence genes (*eaeA*, *hlyA*, stx_1, stx_2 and stx_2 variants) were investigated in this study. Interestingly, the *bfpA* gene was not detected in all the *E. coli* isolates analysed and thus confirmed that the isolates belonged to aEPEC group, an emerging diarrhoeagenic pathogens in both developing and developed countries *(*Beutin *et al.,* 2005; Álvarez-Suárez *et al*., 2016; González *et al*., 2017).

Generally, 350 of the isolates possessed all the primary virulence genes (stx_1, stx_2, *hlyA* and *eaeA*). Similar findings were reported in the previous studies (Momtaz *et al*., 2013a; Momtaz *et al*., 2013b). The *hlyA* gene was the most frequently detected (12.74%) of the isolates among all the virulence genes investigated. These results are consistent with those reported on *E. coli* O157 and O26 serotypes isolated from cattle, beef, vegetable and water intended for human consumption (Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011; Iwu *et al*., 2016; Jajarmi *et al*., 2017). The high prevalence of *hlyA* gene may be attributed to the fact that *hlyA* is a plasmid encoded gene and as a result, it could be transferred between strains of the same species (Ateba and Mbewe, 2011). Since *hlyA* gene encodes α-haemolysin toxin that lyses mammalian erythrocytes, it is worrying that a large proportion of *E. coli* O177 strain harboured this gene (Toro *et al*., 2017).

Another observation was that the *E. coli* O177 isolates possessed *stx* genes. These genes are considered as primary virulence genes in shiga toxin-producing *E. coli* and their presence in aEPEC strains, especially *E. coli* O177, raises a serious public health concern. In this study, a large proportion (11.20%) of the isolates harboured *stx*$_2$ gene fragment. These results are similar those reported in shiga toxin producing *E. coli* strains by the previously studies (Jajarmi *et al*., 2017; Toro *et al*., 2017; Bibbal *et al*., 2018; Cha *et al*., 2018). Interestingly, *stx*$_2$ positive isolates also harboured *stx*$_{2a}$ and *stx*$_{2d}$ gene subtypes. Although other *stx*$_2$ subtypes were not detected in this study, detection of *stx*$_{2a}$ and *stx*$_{2d}$ genes in *E. coli* O177 isolates is an important finding because these genes are associated with HC and HUS infections in humans (Farrokh *et al*., 2013). Furthermore, strain harbouring *stx*$_2$ gene is more virulent than those carrying either *stx*$_1$ or both *stx*$_1$ and *stx*$_2$ genes (Farrokh *et al*., 2013; Toro *et al*., 2017). Therefore, the presence of *stx*$_2$ gene and its subtypes in *E. coli* O177 may augment the virulence of this strain and thus creating a serious public health concern.

The *stx*$_1$ and *eaeA* genes were also detected but at low levels compared to *hlyA* and *stx*$_2$ gene fragments ($p < 0.001$). A small proportion (9.07%) of the isolates possessed *stx*$_1$. These findings are similar to those previously reported in STEC strains (Ateba and Mbewe, 2011; Bai *et al*., 2015). The strain carrying *stx*$_1$ may cause diarrhea in immunocompromised individuals (Farrokh *et al*., 2013). The *eaeA* gene encodes for the outer membrane and thus mediates adherence between the STEC and/or EPEC cells and intestinal epithelial cells (Farrokh *et al*., 2013). Although this gene was detected in this study, some isolates were negative for *eaeA* gene. This could be attributed to the fact this gene, particularly in *E. coli* o177 strain, is weak and hence the reason why some isolates tested negative. These results differ significantly with other studies which detected high *eaeA* in aEPEC strains (Trabulsi *et al*., 2002; Otero *et al*., 2013; Álvarez-Suárez *et al*., 2016; Ingle *et al*., 2016; Martins *et al*., 2016; Ghanbarpour *et al*.,

2017; González *et al*., 2017; Bibbal *et al*., 2018). Given that the strains carrying *eaeA* gene are considered potentially pathogenic, absence of this gene in some of the isolates in this study should not be underestimated. In addition, there is scientific evidence that some *eaeA*-negative strains cause severe diseases in humans (Toro *et al*., 2017). The *eaeA*-negative strains may use other genes such as *saa*, *aidA*, *agn43*, *ehaA* or *iha* to adhere to the epithelial cell of the host (Toro *et al*., 2017). However, the presence of these genes were not investigated in this study.

The co-expression of *stx*$_1$, *stx*$_2$, *hlyA* and *eaeA* genes may increase the pathogenicity of *E. coli* strains (Jajarmi *et al*., 2017). It was observed that *E. coli* O177 isolates possessed combinations of these virulence genes. There were twelve different combinations of virulence genes detected in this study. The most frequently detected combinations were *stx*$_2$/*hlyA*, *stx*$_1$/*stx*$_2$, *stx*$_1$/*stx*$_2$/*hlyA* and *hlyA*/*eaeA* (9.74%, 7.40%, 7.09% and 6.80%, respectively) ($p < 0.001$). While a small proportion (5.11%) of the isolates harboured all the four genes (*eaeA*/*hlyA*/*stx*$_1$/*stx*$_2$), a large proportion (32.7%) of the isolates possessed *stx*$_1$/*stx*$_2$ combination. It was also remarkable that one isolate possessed six virulence genes (*stx*$_1$/*stx*$_2$/*hlyA*/*eaeA*/ *stx*$_{2a}$/*stx*$_{2d}$). This is a significant finding because co-expression of these genes may increase the virulence of *E. coli* O177 strain and thus pose public health concern. These findings are similar to those reported in *E. coli* O26 in a previous studies (Jajarmi *et al*., 2017; Ranjbar *et al*., 2017). However, there was low rate of combination of *stx*$_2$ subtypes as compared to the previous study (Jajarmi *et al*., 2017). Furthermore, the combinations of virulence genes reported in this study are higher than those reported in *E. coli* O157 (Ateba and Mbewe, 2011; Jajarmi *et al*., 2017; Toro *et al*., 2017). This could be attributed to the strain (STEC) investigated and geographical location.

Antimicrobial agents play a vital role in both humans and animals' lives. In animal husbandry, antimicrobial agents are used for various purposes such as treatment of diseased animals or as

growth promoters and/or for prophylactic purpose (Hudson *et al*., 2017). However, overuse of antimicrobial agents in food-producing animals, often without professional consultation and supervision, has precipitated the development of new antibiotic resistance genes such as *mcr* gene encoding for colistin resistance in foodborne pathogens (Liu *et al*., 2016). In addition, antibiotic resistance has led to reduction of the therapeutic potential of most antimicrobial agents used human (Qiao *et al*., 2017). As a result, the use of medically important antimicrobial agents in animals is prohibited in certain jurisdictions. Despite this, farmers continue to use antimicrobial agents to maximise production. South Africa is one of the countries where the usage of antimicrobial agents in animal production is very high (Hudson *et al*., 2017). Several studies detected antimicrobial resistance genes in *E. coli* O157 and non-O157 strains isolated from cattle, beef, pigs, pork, vegetables and water intended for human consumption and this become a public health concern (Ateba and Bezuidenhout, 2008; Ahmed *et al*., 2014; Ahmed and Shimamoto, 2015,). Nevertheless, the data on *E. coli* O177 antimicrobial resistance profiles is limited.

In this study, *E. coli* O177 isolates revealed phenotypic resistance against all the seven classes of antimicrobial agents tested. In contrary to the previous study which reported high prevalence of resistance against ampicillin (100%), gentamicin (100%) and tetracycline (96.87%) Ranjbar *et al*. (2018), this study revealed resistance against erythromycin (63.84%), ampicillin (21.54%), tetracycline (13.37%), streptomycin (17.01%) and kanamycin (2.42%). These disparities could be attributed to geographical location and the sample type. Furthermore, 20.74% of the isolates were resistant to at least three and antimicrobial agents tested. In addition, 20.74% isolates exhibited different multi-drug resistance (MDR). One isolate revealed extensively drug-resistant (XDR) phenotypes to six antibiotics groups tested. The MAR index ranged between 0.29 and 0.86 and the average was 0.65. These results were

similarly to those reported in the previous study (Ranjbar *et al*., 2017). This clearly demonstrate that with time, these antibiotics will be less effective and/or completely ineffective against *E. coli* O177 strain.

Another important observation was that all isolates harboured antibiotic resistance genes Antimicrobial resistance genes encoding for three antibiotics (erythromycin, streptomycin and tetracycline) were detected. It has been reported that antibiotic resistance is common among *E. coli* isolates obtained from animals and food of animal origin due to frequent use of antibiotics in animals (Ryu *et al*., 2012). It is also worth mentioning that the same isolates harboured STEC virulence genes and previous studies have shown that isolates with similar genetic determinants may pose severe complications in humans (Ateba and Bezuidenhout, 2008; Hemeg, 2018). The occurrence of antibiotic resistance genes detected in this study was higher as compared to the previous study (Ryu *et al*., 2012). This could be due to the relatively higher usage of antimicrobial agents in the intensive livestock farming system where the isolates were obtained.

Antibiotic resistance genes associated with some of the antibiotics tested in this study were detected. The aadA, *strA* and *strB* were the most frequently detected genes. However, there were significant differences among all the genes. Most of the isolates possessed *aadA* (22.86%), *strA* (19.85%) and *strB* (19.38%) genes that code for resistance to streptomycin and similar observations have been reported in other studies (Srinivasan *et al*., 2007; Shahrani *et al*., 2014; Bibbal *et al*., 2018). Despite the fact that *tet* genes are commonly found in *E. coli* from cattle due to the high usage of tetracycline in dairy and feedlot cattle, the occurrence of *tet* resistance genes in *E. coli* O177 isolates was low. Only the *tetA* gene was detected individually or in combination with other genes. However, in a previous report (Olowe *et al*., 2013), the proportion of *tet*A (43.8%) was higher than *tetB* (32.0%) and a combination of both

tetA and *tetB* (4.4%) among clinical *E. coli* isolates. In addition, Ranjbar *et al*. (2018) reported *tetA* (76.56%), *tetB* (20.31%), *cat1* (18.75%) and *cmlA* (1.50%) in *E. coli* O157 and non-O157 serogroups from milk. Another study reported high detection of *tetA* and *tetB* in *E. coli* isolated from urinary infection patients, ruminants and donkey raw milk (Momtaz *et al*., 2012; Momtaz *et al*., 2013c). Similar to a previous report (Olowe *et al*., 2013), none of the isolates in this study harboured the *tetW*, *amp*, *cml*A and *kan* genes.

3.5. Conclusion

To the best of our knowledge, this is the first study, to report the occurrence of *E. coli* O177 strain in cattle, especially in South Africa. This study showed that *E. coli* O177 strain harbours virulence genes similar to those found in STEC such as O157 and non-O157 strains. This clearly indicates that aEPEC may contain highly virulence strains. In addition, *E. coli* O177 strain revealed high resistance to aminoglycosides antibiotic group, posing a public health concern. The primers and multiplex PCR assay developed in this study proved to be efficient, specific, sensitive and reproducible in detecting *E. coli* O177 strain from environmental samples. In addition, this protocol can be used in a routine laboratory analysis as well as monitoring *E. coli* O177 strain in food products. It is also fair to state that the use of few antibiotics for antimicrobial susceptibility test is regarded as the limitation of this study. Therefore, whole genome sequencing of *E. coli* O177 is required in order to understand the potential pathogenicity of this strain.

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CHAPTER FOUR

GENETIC RELATEDNESS AND WHOLE GENOME SEQUENCING OF *ESCHERICHIA COLI* O177 STRAIN ISOLATED FROM CATTLE FAECES

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CHAPTER FOUR

GENETIC RELATEDNESS AND WHOLE GENOME SEQUENCING OF *ESCHERICHIA COLI* O177 STRAIN ISOLATED FROM CATTLE FAECES

(This chapter has been submitted for review in the journal "*Environment International*" with authors Peter Kotsoana Montso, Cornelius Carlos Bezuidenhout, Victor Mlambo and Collins Njie Ateba)

Abstract

Atypical enteropathogenic *E. coli* (aEPEC) is a genetically diverse group of foodborne pathogens responsible for causing multistate and multinational outbreaks of foodborne infections in humans worldwide. The need for rapid, sensitive and robust molecular typing methods with high discriminatory power to trace the source of infection during outbreaks is critical. The purpose of this study was to determine genetic relatedness and whole genome sequence (WGS) of multi-drug resistant *E. coli* O177 isolates obtained from cattle faeces from different cattle farming systems in South Africa. A total of 96 *E. coli* O177 isolates were typed using enterobacterial repetitive intergenic consensus (ERIC) and random amplified polymorphism deoxyribonucleic acid (RAPD) PCR analysis. The ERIC-PCR yielded fragments from 93.8% of the isolates and 84 ERIC-typing patterns with 0.95 discriminatory index were generated. ERIC typing grouped the isolates into nine clusters, which contained mixed isolates from different farms. At 95% delineation, 12 isolates from different farms revealed 100% genetic similarities. On the other hand, RAPD PCR analysis revealed consistent and reproducible fragments form 59.4% isolates and 42 RAPD typing patterns with 0.95 discriminatory index were observed. The RAPD typing classified the isolates into eight clusters

composed of isolates from the various cattle farms. *In silico* serotyping search following WGS, showed that the two isolates sequenced were confirmed as O177 strain with H7 (CF-154) and H21 (CF-335). Whole genome sequencing revealed that *E. coli* O 177 harbours several virulence and antimicrobial resistance genes. The key virulence genes such as intimin (*eaeA*), haemolysin (*hlyA* and *hlyE*) and others associated with the aEPEC group were found in both genomes. However, genes (*stx*) encoding for shiga toxins were not found in both genomes. Furthermore, WGS results indicated that both genomes possessed six plasmid types, prophages and cluster of regularly interspaced short palindromic repeats (CRISPR) type I (subtype I-A and I-E). The CRISPR-Cas proteins were found in both genomes. These findings indicated that there is high genetic similarities between *E. coli* O177 isolates obtained from various faming systems. The *E. coli* O177 isolates harbour virulence, antimicrobial resistance and biofilm genes and thus have high potential to cause infections in humans. Therefore, proper hygiene practices and continuous monitoring of *E. coli* O177 strain is imperative for food safety.

***Keywords*:** *E. coli* O177, genetic diversity, ERIC and RAPD typing, whole genome sequencing.

4.1. Introduction

Molecular typing methods have been developed and used to establish genetic relatedness between foodborne pathogens isolated from various sources (Nath *et al.*, 2010; Adzitey *et al.*, 2013; Ateba and Mbewe, 2014). Typing methods facilitate the investigation of outbreaks of foodborne by providing more information regarding the distribution and epidemiology of a particular pathogen. In addition, their application is based on discriminatory power, reproducibility, typing ability, cost effectiveness and interpretation of the results (Adzitey *et al.*, 2013). Against this background, several studies employed typing techniques such as pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST), enterobacterial

repetitive intergenic consensus (ERIC) and random amplified polymorphism deoxyribonucleic acid (RAPD) to assess genetic similarities of food borne pathogens (Nath *et al.*, 2010; Ateba and Mbewe, 2013; Ateba and Mbewe, 2014; Nair *et al.*, 2015).

Despite the fact that molecular typing methods are useful techniques to assess genetic diversity of foodborne pathogens, the combination of typing methods with whole genome sequencing is key when establishing the source infection during outbreaks of foodborne pathogens (Stratakos *et al.*, 2020). Whole genome sequencing (WGS) is a powerful tool with high throughput and discriminatory power, which provides a greater insight into genomic content such as virulence and antimicrobial resistance profile of foodborne pathogens (Jagadeesan *et al.*, 2019). Several public health and food safety agencies such as World Health Organisation (WHO), Food and Drug Administration (FDA) and Centers for Disease Control and Prevention (CDC) are using WGS to characterise foodborne pathogens (Rantsiou *et al.*, 2018).

Atypical enteropathogenic *E. coli* (aEPEC) strains are considered as emerging foodborne pathogens (Xu *et al.*, 2017). This group comprises of heterogeneous strains with divergent virulence factors similar to those found in shiga toxin producing strains (Trabulsi *et al.*, 2002 Croxen *et al.*, 2013). In addition, aEPEC strains are causing severe diarrhoea in all age groups, particularly in humans (Ingle *et al.*, 2016a). Contaminated food and water are known to be the source of most foodborne outbreaks (Ateba and Mbewe, 2014). Unlike other foodborne pathogens such *E. coli* O157, *Listeria* and *Salmonella*, no scientific data on the clonal distribution of *E. coli* O177 strain in cattle across different farming systems (intensive, semi-intensive and extensive) in the study area. In addition, there is little information on virulence potential and antimicrobial resistance profile of this serotype (Montso *et al.*, 2019). Hence, ERIC-and RAPD-typing methods were used to determine genetic relatedness of *E. coli* O177

strains isolated from cattle faeces obtained from different farming systems (intensive, semi-intensive and extensive). Furthermore, WGS was performed to assess virulence potential and the presence of antimicrobial resistance genes in the *E. coli* O177 genome.

4.2. Materials and methods

4.2.1. Bacterial strain and culture

A total of 96 *E. coli* O177 isolates from cattle faeces obtained from feedlot and dairy farms in the North West province, South Africa, were selected to determine their genetic relatedness analysed using ERIC and RAPD methods. The selection was based on antimicrobial resistance phenotypes, virulence and antibiotic resistance gene profiles and sampling area/location. Stock cultures stored at −80 °C in 20% (v/v) glycerol were used. Prior to use, the isolates were revived on sorbitol MacConkey agar supplemented with 1 mg/L potassium tellurite and aerobically incubated at 37 °C for 24 hours. Fresh colonies were transferred into 15 mL sterile falcon containing 5 mL nutrient broth. The samples were incubated at 37 °C for 24 hours.

4.2.2. DNA extraction

DNA was extracted from the overnight cultures using ZR Genomic DNA™-Tissue MiniPrep Kit (Zymo Research Corp, Irvine, CA, USA) according to the manufacturer's instructions. The DNA quality and quantity was determined using Nanodrop™-Lite spectrophotometer (Thermo Scientific, Walton, MA, USA) prior to use for molecular typing.

4.2.3. ERIC and RAPD typing analysis of *E. coli* O177 isolates

A total of 96 DNA samples from *E. coli* O177 isolates were subjected to ERIC and RAPD analysis to determine their genetic similarities. The oligonucleotide primer sequences used were synthesised by Inqaba Biotechnical Industry Ltd., Pretoria, South Africa, and single

primer sequences ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') (Ateba and Mbewe, 2014) and 1283 (5'-CCGCAGCCAA-3') (Nath *et al.*, 2010) were used for ERIC and RAPD PCR, respectively. The PCR reaction mixtures were prepared as standard in 25 µL that constituted 12.5 µL of 2x Tag Green Mastermix, 11 µL nuclease free PCR water, 0.5 µL primer and 1 µL of DNA template. A reaction mixture without the DNA template was used as negative control. All the reagents were Biolab (New England, UK) products supplied by Inqaba Biotechnical Industry Ltd., Pretoria, South Africa. Amplification was performed using a DNA thermal cycler (Model-Bio-Rad C1000 Touch™ Thermal Cycler) with initial denaturation at 95°C for 2 minutes, 30 cycles of 94°C for 3 seconds, 50°C for 1 minute, 65°C for 8 minutes and final elongation at 65°C for 8 minutes for ERIC PRC. The PCR conditions used for RAPD were initial denaturation at 95°C for 5 minutes, 45 cycles of 94°C for 3 seconds, 36°C for 1 minute, 72°C for 1 minute and final elongation at 72°C for 10 minutes. The amplicons were stored at 4 °C until electrophoresis was performed. The amplicons were resolved by electrophoresis on a 1% (w/v) agarose (Seakme®, Rockland, USA) gel (stained with 0.5 µg/mL) using 1x Tris-acetate–EDTA (40 Mm Tris, 1mM EDTA and 20mM glacial acetic acid; pH 8.0) on a horizontal agarose gel equipment (Bio-Rad). A 1 kb DNA molecular weight ladder (Fermentas, Glen Burnie, USA) was included in each gel to determine the size of the amplicons.

4.2.4. Analysis of the gel images

The images were captured with ChemiDoc imaging system (Bio-Rad ChemiDoc™ MP imaging system, UK) using the GeneSnap (version 6.08) software. Digital images of both typing methods were converted to Tagged Imaging File format (TIFF) and analysed using BioNumerics Software (version 5.1; Applied Maths, Sint-Martens-Latem, Belgium). A standard molecular weight ladder (1 kb Plus DNA Ladder, #N3232S, supplied by Inqaba Biotechnical Industry Ltd., Pretoria, South Africa) included in the gel was used for

standardising the images. The ERIC and RAPD bands patterns were compared using Dice coefficient method with optimisation of 0.5% and band position tolerances set at 1%. Cluster analysis was performed using unweighted pair group method with arithmetic mean (UPGMA) to construct dendrograms. The numerical index of the discriminatory power of ERIC-and RAPD-PCR was assessed by applying Simpson's diversity index of diversity equation as previously described (Hunter and Gaston, 1988). The following formula was used:

$$\mathrm{D} = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1)$$

where N is the total number of isolates in the samples population, s is the total number of types described, and n is the number of isolates belonging to the j^{the} type. The Simpson's diversity index ranges from 0.0 to 1.0, where a value of 0.0 indicates no discriminatory while 1.0 shows high discriminatory power.

4.2.5. DNA extraction for whole genome sequence analysis

Two *E. coli O177* isolates (CF-154 and CF-335) were selected for whole genome sequence. DNA was extracted from the overnight cultures using ZR Genomic DNA^{TM}-Tissue MiniPrep Kit (Zymo Research Corp, Irvine, CA, USA) according to the manufacturer's instructions. The genomic DNA was purified using Norgen phage DNA isolation kit (Norgen Bioteck Corp., Ontario Canada), following the manufacturer's instructions. The DNA purity and quantity was measured using Nanodrop^{TM}-Lite spectrophotometer (Thermo Scientific, Walton, MA, USA). Purified DNA sample was sequenced at the Department of Molecular Microbiology, North West University, Potchefstroom Campus.

4.2.6. Shotgun sequencing

Bacterial DNA (from CF-154 and CF-335 isolates) sample was sequenced at Molecular Microbiology Laboratory, NWU, Potchefstroom Campus. Briefly, bacterial DNA was fragmented using ultrasonication approach (Covaris) and resulting DNA fragments were size selected (300-800bp) using AMPure XP beads, end repaired and Illumina specific adapter sequences ligated. DNA sample was diluted to a standard concentration (4nM) before sequencing on Illumina's MiSeq platform, using a MiSeq v3 (600 cycle) kit, following the manufacturer's standard protocol which generated 576.5 Mb (CF-154's genome) and 794.3 Mb (CF-334's genome) of data (2x300 bp long paired-end reads) per sample.

4.2.7. Quality and trimming

Raw sequence data was uploaded into Kbase platform (v2.1) (https://kbase.us/) (Arkin *et al.*, 2018). The quality control of the raw reads was performed using FastQC (v0.11.5) (Andrews, 2010). The adaptors and ambiguous nucleotides sequences were filtered and trimmed using Trimmomatic (v0.36) at default parameters (low quality sequence: 0.05 and ambiguous nucleotides: maximal 2 nucleotides allowed) (Bolger *et al.*, 2014). Reads shorter than 50 nucleotide bases (0.6% for CF-154's genome and 0.6% for CF-335's genome) were removed. The cleaned raw data of these two samples were deposited into https://www.ncbi.nlm.nih.gov/ to obtain the sequence reads archive (SRA) accession numbers; PRJNA554852 and PRJNA555014 (CF-334's genome and CF-154's genome, respectively).

4.2.8. *De novo* assembly and annotation

The *de novo* assembly of the trimmed (good quality) reads was performed using SPAdes v3.13.0 (Bankevich *et al.*, 2012). *De novo* options were minimum contig length, minimum size of the assembled contigs of 500 bp and 1000 bp, respectively. *De novo* assembly of the trimmed

reads was carried out using the following parameters: similarity = 0.8, length fraction = 0.5, insertion cost = 3, deletion cost = 3, mismatch cost = 3, auto-detect pared distance and performed scaffolds. Gene prediction and genome annotation were performed using Prokaryotic Genome Annotation Pipeline (PGAP) (Zhao *et al.*, 2011) and Rapid Annotation using Subsystem Technology (RAST v2.0) server (Aziz *et al.*, 2008).

The genomes were searched for serotype, virulence and antibiotic resistance genes using Sero-TypeFinder version 2.0 (Joensen *et al.*, 2015), VirulenceFinder version 2.0 (Joensen *et al.*, 2014) and ResFinder version 2.2 (Zankari *et al.*, 2012), respectively (all at 95% ID threshold), which are online tools developed by the Center for Genomic Epidemiology (CGE) (www.genomicepidemiology.org). The presence of plasmid types were identified by PlasmidFinder 1.3 (https://cge.cbs.dtu.dk/services/PlasmidFinder/). The presence of prophage sequences were assessed using Phage Search Tool Enhanced Release (PHASTER) http://phaster.ca/. The genomes were searched for intact, questionable and incomplete prophages sequences (> 90, 70-90 and < 70 threshold, respectively) (Zhou *et al.*, 2011; Arndt *et al.*, 2016). The CRISPRone (http://omics.informatics.indiana.edu/CRISPRone) tool was used to determine the presence of Cluster of regularly interspaced short palindromic repeats (CRISPR-Cas system) in both genomes. The types and subtypes of CRISPR-Cas system, number of loci, length and nucleotide sequences of repeats spacers, CRISPR-Cas system genes and proteins were searched (Zhang and Ye, 2017). The genome sequences of both isolates were deposited into the GenBank under the accession numbers, VMKG00000000 and VMKH00000000 (CF-334's genome and CF-154's genome, respectively). The genome maps were drafted using Pathosystems Resource Integration Center (PATRIC, version 3.5.43) on line tool (https://patricbrc.org/).

4.3. Results

4.3.1. Enterobacterial repetitive intergenic consensus (ERIC) PCR analysis

A total of 96 antimicrobial resistant *E. coli* O177 isolates obtained from cattle faeces from different commercial farms were typed using ERIC techniques. ERIC PCR typing generated stable and reproducible bands with different sizes from 90 (93.8%) *E. coli* O177 isolates. The number of amplicons generated by ERIC PCR ranged from 4 to 12 bands per isolate, Figure 4.1. A larger proportion (73.3%) of the isolates displayed at least seven and more fragments while the smaller proportion (26.7%) yielded six DNA fragments per isolate. The maximum number of fragments yielded per isolate was 12 bands per isolate. The bands size ranged from 0.1 to over 15.0 kb. A common band at 0.55 kb was found in 93.3% of *E. coli* O177 isolates.

Based on ERIC typing pattern, 90 *E. coli* O177 isolates were differentiated into 84 ERIC types, Figure 4.2. These isolates were grouped into nine clusters (designated I - IX) at ≥ 35% similarity coefficient. At delineation level of ≥ 95% genetic similarity, only 35 isolates were grouped into 15 clusters and the remaining 55 isolates were placed separately. At delineation level of ≥ 95%, twelve isolates were undistinguishable revealing 100% genetic similarity, Figure 4.3 (cluster II, VI, VII and IX). One pair of the nontypeable isolates were obtained from the samples collected from CF-F-D and CF-G farm while the remaining five pairs were obtained from the samples from the same farms (CF-C = 1 pair, CF-D-D = 1 pair, CF-E = 2 pairs and CF-F-D = 1 pair). The clusters were further analysed based on source (farm), Table, 4.1. Clusters I, II and IX had 17, 23 and 21 isolates, respectively, while clusters III, IV and VII comprised of three isolates each. All the clusters contained mixed isolates from more than one farm.

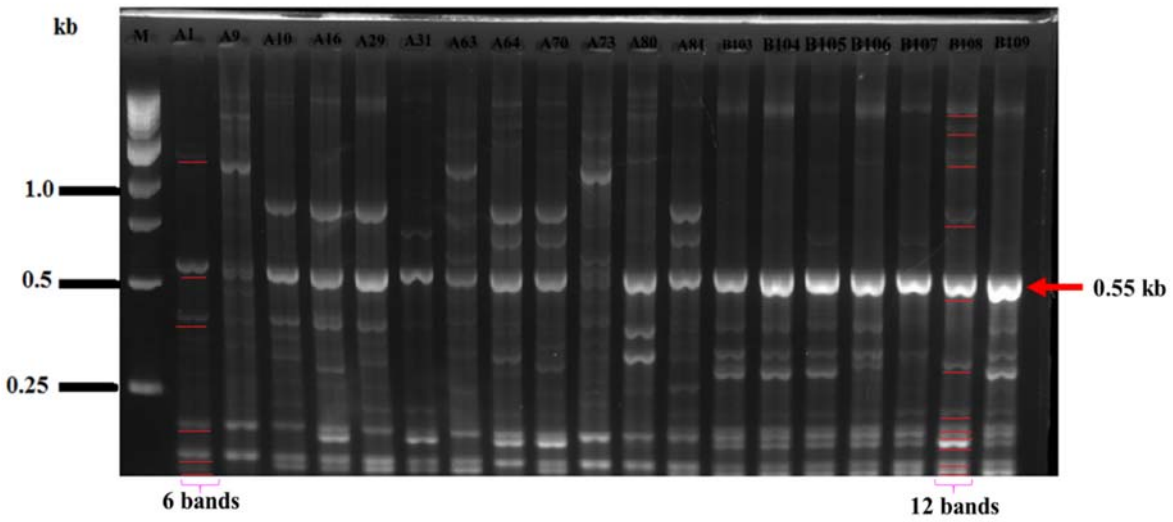

Figure 4.1: Agarose gel [2%] image depicting ERIC profiles of representative *E. coli* O177 isolates obtained from cattle faeces from different farms. Lane M, 1 Kb Plus DNA ladder; Lane A1-B109; *E. coli* O177 isolates from cattle faeces.

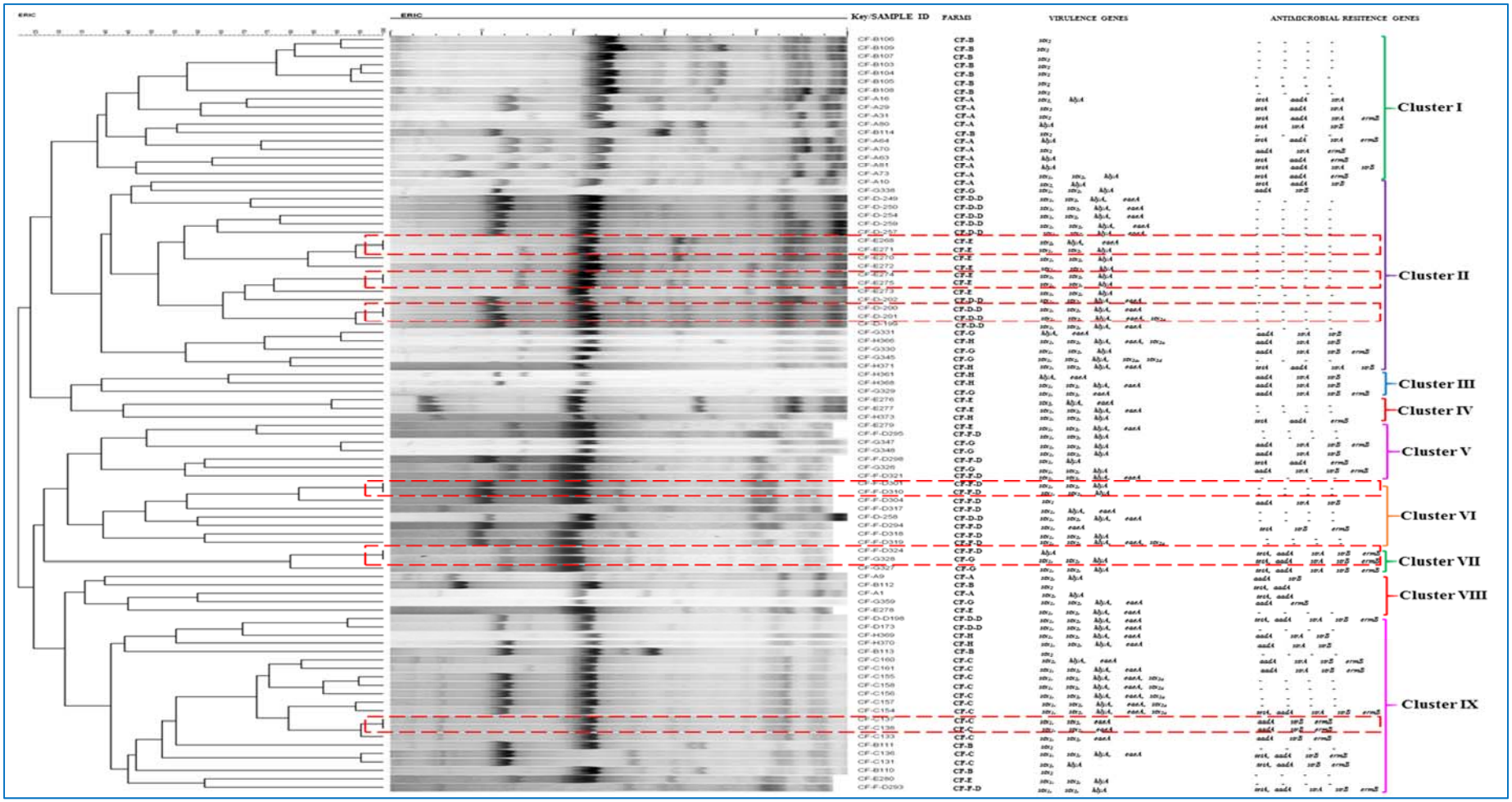

Figure 4.2: Dendrogram showing genetic relatedness of *E. coli* O177 isolated from different farms as determined by ERIC-PCR fingerprinting technique. The isolates showing identical ERIC pattern are marked in broken lines red rectangles. The similarities between the patterns was calculated from Dice Index.

Table 4.1: ERIC cluster patterns of *E. coli* O177 isolates from different commercial farms.

Cluster	ERIC pattern	Farm	Number of isolates
I	17	CF-A, CF-B	17
II	20	CF-A, CF-E, CF-D-D, CF-G, CF-H	23
III	3	CF-E, CF-G, CF-H	3
IV	3	CF-E, CF-H	3
V	7	CF-E, CF-F-D, CF-G	7
VI	7	CF-D-D, CF-F-D	8
VII	2	CF-F-D, CF-G	3
VIII	5	CF-A, CF-B, CF-G, CF-E	5
IX	20	CF-B, CF-C, CF-D, CF-E, CF-F-D, CF-H	21

4.3.2. Random amplification of polymorphic DNA (RAPD) PCR analysis

A total of 96 antimicrobial resistant *E. coli* O177 isolates were typed using RAPD PCR analysis to determine their genetic relatedness. RAPD PCR analysis generated stable and reproducible fragments from 60 (62.5%) isolates. RAPD PCR generated 4 to 6 bands per isolates, Figure 4.3. The size of fragments ranged from 0.4 to 15.0 kb. Majority (61.4%) of the isolates revealed four bands while 38.6% isolates showed five and more fragments. A larger (78.9%) proportion of the isolates showed a common band at 0.9 kb, Figure 4.3.

As displayed in Figure 4.4, 57 *E. coli* isolates produced consistent band pattern, which were detectable by BioNumerics Software and 42 RAPD types were generated. These typed isolates were grouped into eight clusters at ≥ 35% similarity coefficient. At a delineation level of ≥ 85% similarity coefficient, 30 isolates were grouped into 11 clusters while the remaining 27

were placed separately. Twenty-six isolates revealed 100% similarities at the delineation level of ≥ 95%, Figure 4.4 (Cluster I, II, IV, V and VI). Only three pairs of undistinguishable types were composed of isolates from samples obtained from different farms. All the clusters contained mixed isolates from different farms, Table 4.2. Clusters I and VI contain 10 and 22 isolates, respectively, while cluster III included 2 isolates from two farms.

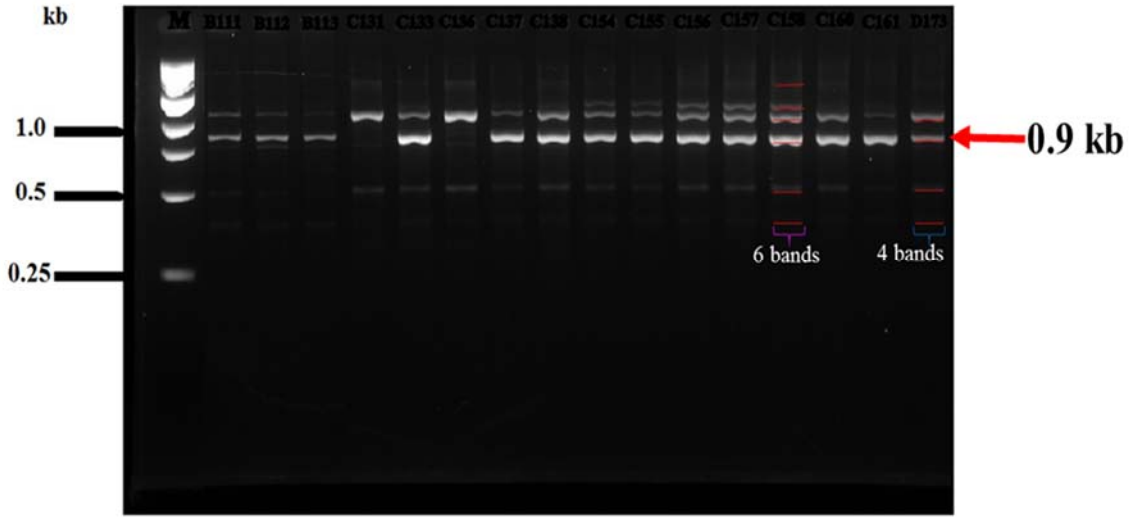

Figure 4.3: Agarose gel [2%] image depicting RAPD profiles of representative *E. coli* O177 isolates obtained from cattle faeces from different farms. Lane M, 1 Kb Plus DNA ladder; Lane A1-D173; *E. coli* O177 isolates from cattle faeces.

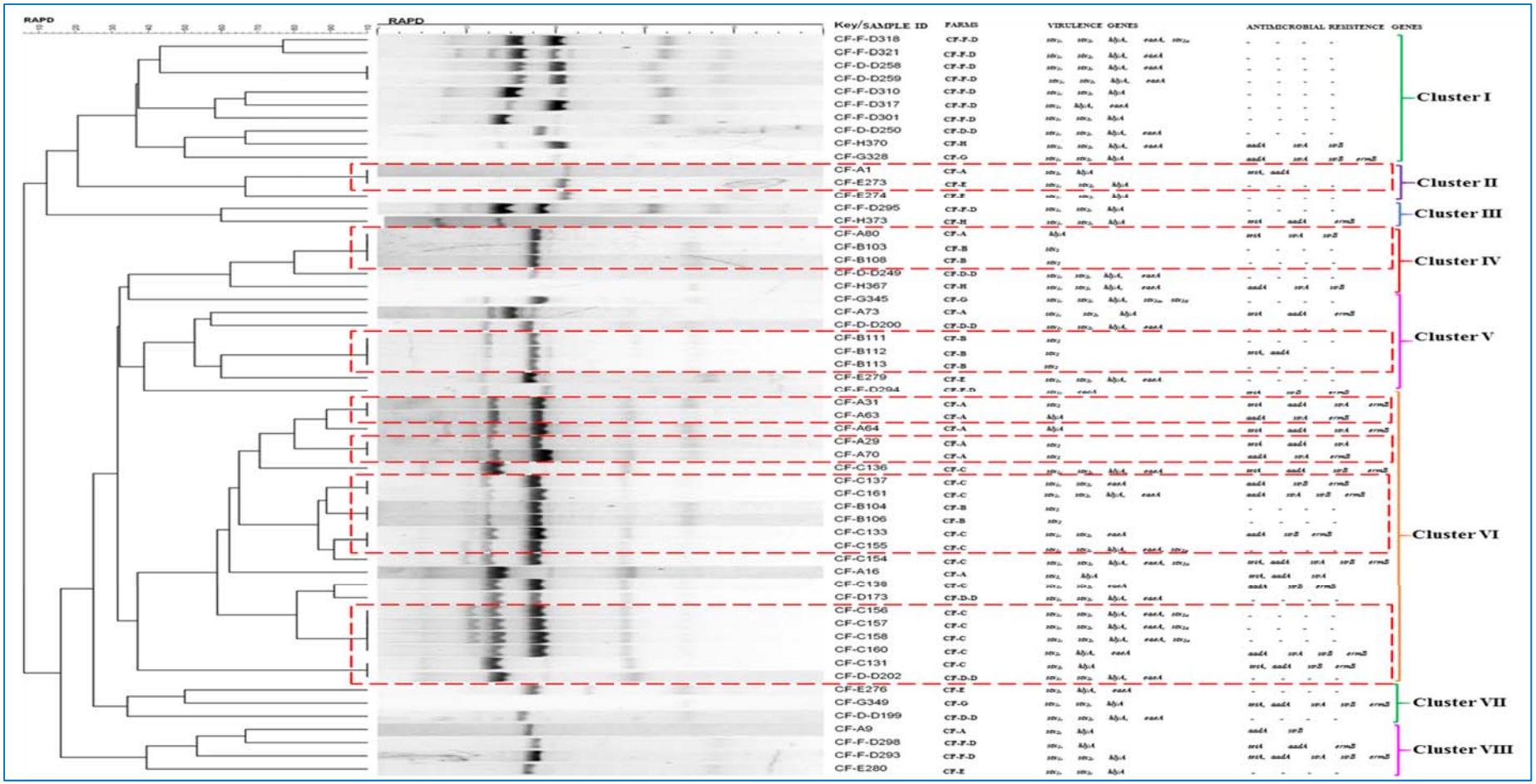

Figure 4.4: Dendrogram showing genetic relatedness of *E. coli* O177 isolated from different farms as determined by RAPD fingerprinting technique. The isolates showing identical RAPD pattern are marked in broken lines red rectangles. The similarities between the patterns was calculated from Dice Index.

Table 4.2: RAPD cluster patterns of *E. coli* O177 isolates from different commercial farms.

Cluster	RAPD pattern	Farm	Number of isolates
I	9	CF-F-D, CF-G, CF-H	10
II	2	CF-A, CF-E	3
III	2	CF-F-D, CF-H	2
IV	4	CF-A, CF-B, CF-D-D, CF-G, CF-H	6
V	5	CF-A, CF-B, CF-D-D, CF-E, CF-F-D	7
VI	13	CF-A, CF-B, CF-C, CF-D, CF-D-D	22
VII	3	CF-D-D, CF-E, CF-G	3
VIII	4	CF-A, CF-E, CF-F-D	4

4.3.3. Genomic characteristics of *E. coli* O177 strain

The Shotgun sequencing analysis was performed to characterise the genomes of two *E. coli* O177 isolates (CF-154 and CF-335) using Illumina's MiSeq platform. The genome coverages for each isolate were 124.7x and 162.128x (CF-154's genome and CF-335's genome, respectively). The genome sizes were 5,112,402 bp and 5,460,435 bp with number of contigs 101 and 191 (CF-154's genome and CF-335's genome, respectively) Table 4.3. The GC content for each genome was 50.7% (CF-154's genome) and 50.5% (CF-335's genome). The genomes contained 5146 and 5570 total genes with 4896 and 5230 coding genes (CF-154's genome and CF-335's genome, respectively). The total protein coding genes were 4896 for CF-154's genome and 5230 for CF-335's genome. The two isolates were identified as human pathogens (PathogenFinder). The Sero-TypeFinder revealed 99% (*wzx* and *wzy* genes) similarity to O177 strain and 100% (*Flic* gene) similarity to H7 (CF-154's genome) and H21 (CF-335's genome). The ResFinder revealed that both genome possessed several antibiotic resistance genes.

The genome annotation using RAST and Patric revealed several virulence, antimicrobial resistance and plasmids encoding genes, Figure 4.5 and 4.6. Thirty-seven genes encoding for the virulence factors were searched and only 94.6% (35/37) were found using both RAST and Patric softwares, Table 4.4. Even with the PCR analysis, *bfp* gene was not found in both genomes. All the virulence genes detected by PCR (*eaeA* and *hlyA*) were found except for *stx* genes, Table 4.4. Some of the virulence genes that were not screened by PCR were also found. A large (94.6%) proportion of the virulence genes were found in CF-154's genome while 89.2% were found in CF-335's genome. The genomes were also annotated to determine the presence of genes encoding for antimicrobial resistance plasmid types and prophages. Twenty-eight antimicrobial resistance genes were searched and 85.7% of them were found, Table 4.5. Two of these genes (*tetA* and *strB*) were detected using PCR. A large (85.7%) proportion of the genes encoding for antimicrobial resistance were found in CF-335's genome while 46.4% were found in CF-154's genome. The genomes carried six plasmid types and prophages sequences, Table 4.6. Both genomes carried 10 prophage regions with five and four intact prophage sequences (CF-154 and CF-335, respectively). The CF-154's genome possessed four incomplete and one questionable sequences while CF-335 harboured three incomplete and three questionable prophage sequences with GC content 50.66% and 50.53% (CF-154's genome and CF-335's genome, respectively).

The CRISPR-Cas system found in both genomes was type 1 (subtype 1-A and 1-E), Table 4.7. The CF-154's genome carried two loci while 335's genome contained one CRISPR locus. Total number nucleotide sequence, average length of repeats and space were highly conserved in both genomes. The two genomes have the same number of average length of repeats and spacers (29 and 32 bp, respectively).

Table 4.3: General genome features of *E. coli* O177 strain isolated from cattle faeces.

Features	**Sample ID**	
	CF-154	CF-335
Genome size	5,112,402 bp	5,460,435 bp
Number of contigs	101	191
Total length	5111092 bp	5459908 bp
GC content (%)	50.7	50.5
N50	127249	113919
L50	14	15
Coding genes	4896	5230
Total genes	5146	5570
Total CDSs	5040	5463
Total proteins	4896	5230

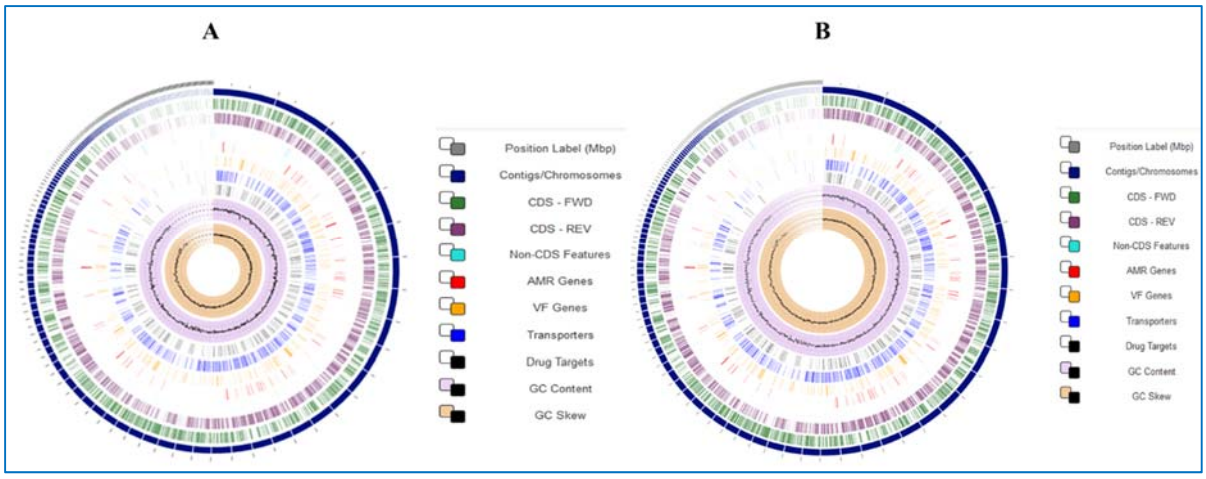

Figure 4.5: The circular genome map of *E. coli* O177 strain (**CF-154-A** and **CF-335-B**). Circle displays from inside to outside: GC Skew (light orange), GC content (light purple), Drug Tagets (black), Transporters (blue), Virulence factor genes (yellow), Antimicrobial resistance genes (red), Non CDS features (turquoise blue), CDS reverse strand (light purple) and CDS forward strand (green).

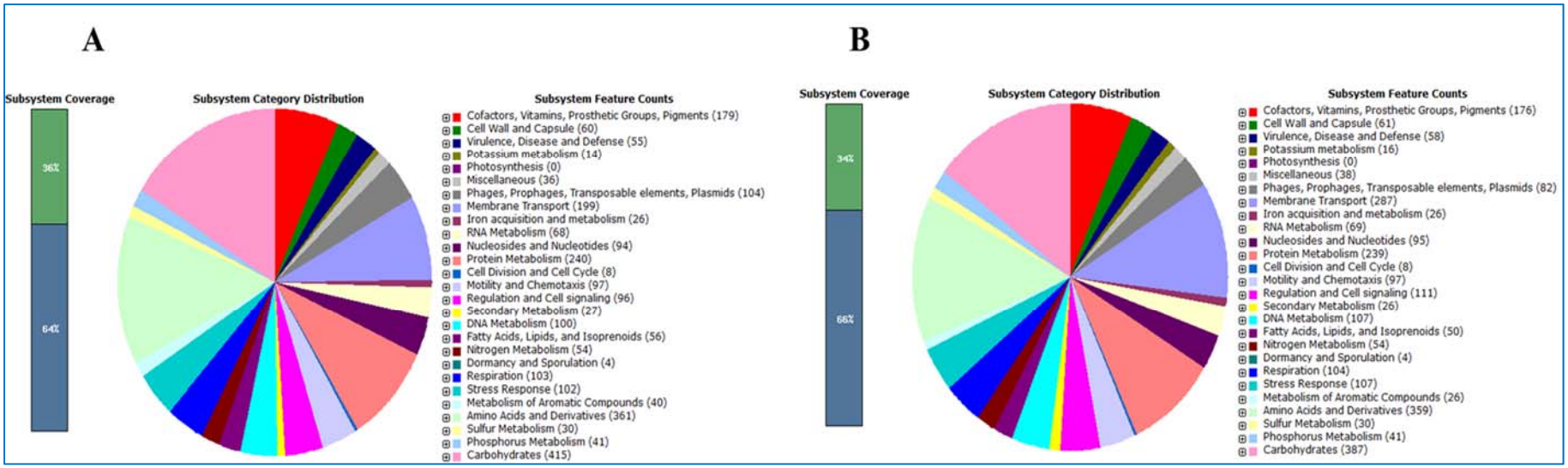

Figure 4.6: *E. coli* O177 strain genome features connect to subsystem and their distribution in different categories (**CF-154-A** and **CF-335-B**).

Table 4.4: Annotated virulence gene results for *E. coli* O177 strain.

Virulence factors	**Genes**	**Sample ID**	
		CF-154	**CF-335**
Bundle forming pili	*bfp*	-	-
Shiga toxins	*stx*	-	-
Haemolysin	*hlyA*	++*	++*
	hlyE	++	++
Invasin/Intimin	*eaeA*	++*	++*
Autotransporter protein	*espI*, *pet*, *pic* and *sat*	++	++
Serine recombinase	*pinR*	++	++
Serine hydroxymethyltransferase	*glyA*	++	++
Serine acetyltrnasferase	*cysE*	++	++
Toxins	*ghoT* and *yhaV*	++	++
Increased serum survival	*iss*	++	-
Type III secretion system	*eprH, gspL* and *prgH*	++	++
Type IV pili	*hcpA* and *ppdD*	++	++
Iron uptake genes	*fieF*	++	-
Adhesins/Fimbriae	*ecpA, lpfA, FimA, FimB, FimE, FimF, FimG* and *FimH*	++	++
Colicin	*cirA*	++	++
Flagella regulator	*flk*	++	++
Plasmid	*traT* and *trbC*	++	++
Biofilm regulator	*bssR* and *bssS*	++	++
Heat shock	*IbpB* and *hsIT*	++	++

Key*:** The ++ = presence, **++ = genes detected by PCR and **–** = not found.

Table 4.5: Annotated antimicrobial resistance gene results for *E. coli* O177 strain.

Class of antibiotics	**genes**	**Sample ID**	
		CF-154	**CF-335**
Beta-lactams (class C)	*ampC*	++	++
Penicillin	*mrcA, mrcB*	++	++
Streptomycin	*aadA* and *strA*	-	-
	strB	-	++*
Sulphonamide	*sul2*	++	++
Tetracycline	*tetA*	-	++*
	tetB	-	++
	tetD	-	++
	tetR	-	++
	tetW	-	-
Aminoglycoside	*AcrD,*	++	++
	aph(6)-Ic	-	++
	aph(6)-Ib	-	++
	aph(6)-Id	-	++
	aph(3'')-I	-	++
	aph(3'')-Ib	-	++
Macrolide	*Maca* and *MacB*	++	++
Erythromycin	*ermB*	++*	++*
Chloramphenicol	*cmr*	-	++
Fosfomycin	*MdtG*	++	++
Norfloxacin	*MdtH*	++	++
Gentamycin	*ant (3")-1a*	-	-
Kanamycin	*kan*	+	+
Acriflavine protein	*AcrA* and *acrE*	++	++

Key*:** The **++** = presence, **++ = genes detected by PCR and **–** = not found.

Table 4.6: Total number plasmid types and prophage sequences in genome of *E coli* O177 strain.

Sample ID	Plasmids types	Number of prophages regions	Completeness of prophages sequences*			
			Intact	Incomplete	Questionable	GC content (%)
CF-154	IncFIB (AP001918), IncX1, IncFII, IncFII (pCoo), IncN and IncNQ	10	5	4	1	50.66
CF-335	IncFIB (AP001918), IncX1, IncFII, IncFII (pCoo), IncN and IncNQ	10	4	3	3	50.53

***Key*:** *denotes prophage sequence classification using PHASTER scores > 90, 70-90 and < 70 (intact, questionable and incomplete, respectively).

Table 4.7: Annotation of CRISPR-Cas system in *E. coli* O177 isolated from cattle faeces.

Sample ID	CRISPR subtypes	Cas proteins	No. of locus	Number of repeats	Average length of repeats	Number of spacers	Average length of spacers	Locus range (bp)
CF-154	1-A, 1-E	cas1, cas2, cas3, cas5, cas7, csa3, cas6e, cas8e, cse2gr11 and DEDDh	2	(Locus 1) 15	(Locus 1) 29	(Locus 1) 14	(Locus 1) 32	28931-29815
				(Locus 2) 18	(Locus 2) 29	(Locus 2) 17	(Locus 2) 32	55515-56580
CF-335	1-A, 1-E	cas1, cas2, cas3, cas5, cas7, csa3, cse2gr11, cas6e, cas8e and DEDDh	1	17	29	16	32	201496-202500

4.4. Discussion

Molecular typing techniques are widely used to determine genetic relatedness of foodborne pathogens isolated from different sources and/or geographical areas (Foley *et al.*, 2009). The data obtained may provide robust information for tracking and tracing the primary source of infection during outbreaks of foodborne pathogens (Foley *et al.*, 2009; Ateba and Mbewe, 2014). Against this background, several molecular typing methods such as BOXAIR-, ERIC-, MLST-, PFGE- and RAPD-typing have been extensively used either singly or in combination to determine genetic similarities of foodborne pathogens such as *E. coli* O157, non O157 serotypes and *Salmonella* species isolated from different sources (Sabat *et al.*, 2013; Ateba and Mbewe, 2014; Bai *et al.*, 2015). In addition, the results obtained from some of these studies revealed that ERIC-and RAPD-typing techniques discriminated isolates with close genetic similarities and thus suggested that these methods may be a useful tool for epidemiological investigation and surveillance of foodborne pathogens (Ateba and Mbewe, 2014). However, no study has been conducted, using this same approach to determine genetic similarities of *E. coli* O177 isolates obtained from cattle in South Africa.

In this study, two molecular typing methods, ERIC- and RAPD-PCR were employed to determine genetic relatedness of 96 *E. coli* O177 isolates obtained from different farms in the North West province, South Africa. Selection of the methods was based on affordability, easy to use and discriminatory index (Nath *et al.*, 2010; Ateba and Mbewe, 2014). The ERIC PCR analysis produced distinct band patterns ranging between 0.1 to > 15.0 kb in size from the isolates. A large proportion (73.3%) of the isolates displayed at least seven DNA fragments. In addition, the ERIC PCR analysis yielded 84 typing patterns with 0.95 discriminatory index and 93.8% reproducibility. A similar observation was reported in other studies, where ERIC typing of *E. coli* O157 and *Salmonella* species isolated from different sources revealed 0.943 to 0.982

discriminatory index with 100% reproducibility (Nath *et al.*, 2010; Ateba and Mbewe, 2014; Nair *et al.*, 2015). Notwithstanding the high discriminatory power of PFGE and MLTS typing methods (Foley *et al.*, 2009), the results presented in this study indicate that ERIC typing is the best tool for typing foodborne pathogens, particularly *E. coli* O177.

Based on ERIC typing pattern, the *E. coli* O177 isolates were grouped into nine different clusters composed of mixed isolates from different farms. Interestingly, isolates from different farms revealed 100% genetic similarities. A similar observation was reported in a previous study (Ateba and Mbewe, 2014). Furthermore, it is also worth mentioning that *E. coli* O177 isolates, showing close genetic similarities possessed similar virulence and antibiotic resistance gene profiles. This demonstrates clonal distribution of *E. coli* O177 strain in cattle across different farming systems. Therefore, poor farm management and hygiene practices may precipitate the spread of the *E. coli* O177 strain.

Although 96 isolates were subjected to RAPD-PCR analysis, only 60 isolates produced stable and reproducible bands. Inconsistency and low reproducibility indicated that primer 1283 is not the best choice for RAPD-PCR analysis, especially for typing *E. coli* O177 strain. A similar observation was reported in a previous study (Nath *et al.*, 2010), which revealed 40% reproducibility on *Salmonella* species using RAPD. The RAPD-PCR analysis yielded bands with different sizes, ranging between 0.4 and 15.0 kb. In addition, a large proportion (61.4%) of RAPD typed isolates revealed four bands while 38.6% isolates showed five or more fragments.

Based on RAPD typing patterns, the isolates were grouped into eight clusters. The clusters were composed of isolates from different farms. Furthermore, isolates from different farming

systems shared genetic similarities such as virulence and antimicrobial resistance gene profiles in agreement with the results obtained from ERIC typing methods. Although a small number of isolates were typed using RAPD methods, discriminatory index of this typing method was 0.859. These results are consistent with reports from previous studies (Nath *et al.*, 2010; Nair *et al.*, 2015). However, poor reproducibility of the primer (1283) presents a serious drawback for the use of RAPD methods (Foley *et al.*, 2009).

The use of next generation sequence (NGS) technology such as whole genome sequencing (WGS) may enhance knowledge and allow better understanding of the genomic content of foodborne pathogens (Kwong *et al.*, 2015). Whole genome sequencing has revitalised food safety and medical industries by enabling high-resolution identification and typing of foodborne pathogens (Jagadeesan *et al.*, 2019). In addition, platforms such as Illumina, Pacific Biosciences and Oxford Nanopore are the most common technologies used for the NGS (Jagadeesan *et al.*, 2019). Moreover, sophisticated omics-based tools are employed to annotate microbial genome and to determine the potential of pathogenicity and antimicrobial resistance of different pathogens. Several studies have utilised WGS for annotation of unknown genes and prediction of their function using different *in silico* bioinformatics tools (Aziz *et al.*, 2008; Zhou *et al.*, 2011).

In this study, two genomes of *E. coli* O177 isolates (CF-154 and CF-335) were sequenced using Illumina's MiSeq platform to determine pathogenicity and antimicrobial resistance gene profile of the aEPEC O177 strain. The sizes of the two genomes were 5,112,402 bp (CF-154) and 5,460,435 bp (CF-335) with GC content 50.7% and 50.5%, respectively. In addition, a total of 5146 and 5570 genes (CF-154 and CF-335, respectively) were found in each genome with respective total proteins of 4896 and 5230. The genome sizes were higher than that of *E. coli*

K strain (Blattner *et al.*, 1997; Hayashi *et al.*, 2001). The data was submitted into Center for Genomic Epidemiology database to confirm the identities of the two genomes. The lipopolysaccharides (O) and flagella (H) are surface antigens of *E. coli* species (Ingle *et al.*, 2016b). The O-antigen is a highly conserved variable region, which contains gene clusters such as *rmlB*, *rmlD, wzy* and *wzx* genes (Debroy *et al.*, 2016). These genes, together with *Flic* gene encoding for the flagellar antigen, are used to identify and differentiate pathogenic *E. coli* strains. Sero-TypeFinder revealed 99% similarity to O177 strain (*wzx* and *wzy* genes) and 100% similarity to H7 and H21 (*fliC* gene) for CF-154's genome and CF-335's genome, respectively. This confirmed that both isolates were *E. coli* O177 strain.

The genomes were further annotated for the presence of virulence genes encoding for bundle forming pili (*bfp*) shiga toxins (*stx*), haemolysis (*hlyA*), intimin (*eaeA*) and other genes associated with aEPEC infections. It was observed that both genomes contained several virulence genes associated with aEPEC pathogens. Interestingly, *bfp* operon was not found in both genomes and this was consistent with the results obtained from PCR analysis. The absence of *bfp* operon confirmed that the two isolates belong to the aEPEC group (Trabulsi *et al.*, 2002). The other virulence genes found in both genomes were similar to those found in other *E. coli* groups such as enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), diffusely adherent *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC) and uropathogenic *E. coli* (UPEC) (Hernandes *et al.*, 2009; Abreu *et al.*, 2013; Xu *et al.*, 2017). This indicates that aEPEC is a highly heterogeneous group with diverse virulence factors (Croxen *et al.*, 2013). Interestingly, a rare *hlyE* gene, which has haemolytic activity on mammalian red blood cells, was also found in both genomes. The combination of co-expression of *hlyA* and *hlyE* and other virulence genes in *E. coli* O177 may render this serotype even more virulent with possible repercussions for human health. Although stx_1, stx_2, stx_{2a} and stx_{2d} were detected using PCR analysis, none of

these genes was found in both genomes. Similar results were reported in a study by Bumunang *et al.* (2019). This could be the result of the repetitive culturing process leading to loss of *stx* genes (Karch *et al.*, 1992). In addition, *stx* genes are encoded in prophages, which are highly unstable, possibly resulting in the loss of *stx* operon by the hosting bacteria (Krüger *et al.*, 2018). However, this mechanism is poorly understood and the genetic phenomena governing *stx* gene loss requires further investigation (Bielaszewska *et al.*, 2007).

Antimicrobial resistance is a serious global threat to veterinary and public health (Zhou *et al.*, 2018). The two genomes were annotated for the presence of antimicrobial resistance gene determinants. The WGS annotation showed that both genomes harboured several antimicrobial resistance genes, which confer resistance to antibiotics belonging to 13 different groups. These findings demonstrate that *E. coli* O177 strain is a multi-drug resistance pathogen and this may present a major public health concern. Comparatively, a higher number of antimicrobial resistance genes were found in CF-335's genome compared to CF-154's genome. Interestingly, *ampC beta-lactams* (class C) gene, which confers resistance to cephalosporins, cephamycin, oxyimino-cephalosporins and monobactams antibiotics (Jacoby, 2009), was found in both genomes. Other genes such *strB*, *tetA* and *ermB*, which were detected using PCR were found in CF-335's genome. However, *aadA* and *strA* genes were not found in both genomes. This could be attributed to the fact that most antimicrobial resistance genes are located on plasmids and may not be found on the genome or chromosome (Guo *et al.*, 2019). This can be confirmed by plasmid typing approaches such as whole plasmid sequencing (Carattoli *et al.*, 2014).

Plasmids are genetic elements, which play pivotal role in horizontal transfer of genetic materials such as virulence and antimicrobial resistance genes between foodborne pathogens of the same and/or different species (Foley *et al.*, 2009; Carattoli *et al.*, 2014). Furthermore,

acquisition of plasmids harbouring virulence and/or antimicrobial resistance genes may drastically alter the prevalence of virulence and multi-drug resistant pathogens (Carattoli *et al.*, 2014). Against this background, *E. coli* O177 genome was annotated to determine the presence of plasmid types. The WGS indicated that both genomes contained six plasmid types. The plasmids types found in both genomes are known to harbour antibiotic resistance genes such as $bla_{TEM\text{-}1B}$, $bla_{CMY\text{-}2}$, *dfrA8*, *strA*, *strB*, *sul2, tetA* and *tetB* (IncFII, IncFIB, IncFII (pCoo) and IncFIB (AP001918)), *catA1*and *floR* (IncNQ) and $bla_{TEM\text{-}1B}$, $bla_{TEM\text{-}52B}$, $bla_{CTX\text{-}M\text{-}1}$, and $bla_{CTX\text{-}M\text{-}55}$ (IncX1) (Zhang *et al.*, 2018; Salinas *et al.*, 2019). It is also worth mentioning that IncX plasmid group is highly syntenic and has evolved into four successful and a divergent subgroups (Johnson *et al.*, 2012). In view of this, infections caused by *E. coli* O177 strain may have serious effects, particularly in immunocompromised individuals. In addition, these plasmids may accelerate and exacerbate transfer of genetic elements between *E. coli* O177 strain and other bacteria species living in the same niche.

Cluster of Regularly Interspaced Short Palindromic Repeats (CRISPER) is a genetic element found in bacteria. The CRISPER provide adaptive mechanisms to bacteria against foreign DNA, especially phages and/or plasmids (Labrie *et al.*, 2010). It is composed of 21-48 bp direct repeats separated by 26-72bp non-repetitive spacers (Fu *et al.*, 2017). In addition, the 5′ edge of the loci is flanked by a number of *Cas* genes (4-20 in number) in bacterial strains (Sharma *et al.*, 2017). The presence of CRISPER genes encoding Cas proteins, which belongs to different CRISPER types, has previously been reported (Shridhar *et al.*, 2018).

In this study, WGS annotation indicated that both genomes contained CRISPER arrays and prophage sequences. The CRISPER type1 with subtypes 1-A and 1-E was found in both genomes. Moreover, several CRISPER associated proteins were observed. The CF-154's

genome contains one locus and CF-335's genome has two loci. On average, both genomes contained 29 and 32 bp repeats and spacers, respectively. These findings fall within the range of the CRISPER arrays (Fu *et al.*, 2017). Given that many virulence and antimicrobial resistance determinants are acquired through mobile genetic elements, it is anticipated that a strain with a more active CRISPER system may carry fewer mobile genetic elements such as plasmids, prophage sequences, virulence and antimicrobial resistance genes (Toro *et al.*, 2014). In contrast, this study showed that both genomes contained several plasmid types, virulence and antimicrobial resistance genes. These findings are consistent with other studies, which showed that distribution of virulence, antimicrobial resistance genes and/or plasmids do not correlate with CRISPER content in *E. coli* genome (Toro *et al.*, 2014). It is also worth noting that the presence of prophage sequences in the genome may precipitate integration of lambda phage genome harbouring shiga toxins. Thus, the presence of CRISPER in *E. coli* O177 strain may have little effect on the acquisition and spread of virulence, antimicrobial resistance genes and/or plasmids.

4.5. Conclusion

This is the first comprehensive study to determine genetic relatedness and whole genome sequences of multi-drug resistant *E. coli* O177 isolates from cattle faeces in South Africa. Both ERIC and RAPD-typing methods revealed that isolates obtained from samples from different farming systems possessed high genetic similarities. Furthermore, isolates clustered together harboured similar virulence and antimicrobial resistance profiles. Whole genome sequence revealed that *E. coli* O177 strain is a human pathogen and belongs to the aEPEC group due to its lack of *bfp* operon. In addition, *E. coli* O177 genome carried several virulence and antimicrobial resistance genes as well as different plasmid types, prophage sequences and CRSPR alleles. These findings suggest that *E. coli* O177 is a highly virulent serotype and,

therefore, continuous monitoring of foodborne pathogens such as *E. coli* O177 in food products is vital to ensure food safety.

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CHAPTER FIVE

ISOLATION AND CHARACTERISATION OF LYTIC BACTERIOPHAGES INFECTING MULTI-DRUG RESISTANT SHIGA TOXIN PRODUCING ATYPICAL ENTEROPATHOGENIC *ESCHERICHIA COLI* O177 STRAIN ISOLATED FROM CATTLE FAECES

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Abstract

The increasing incidence of antibiotic resistance and emergence of virulent bacterial pathogens, coupled with a lack of new effective antibiotics, has reignited interest in the use of lytic bacteriophage therapy. The aim of this study was to characterise lytic *E.coli* O177-specific bacteriophages isolated from cattle faeces to determine their potential application as bio-control agents. Spot test and efficiency of plating (EOP) analyses were performed to determine the host range for selected phages. Phage morphotype was analysed using transmission electron microscopy and the one-step growth curve method. Phages were also assessed for thermal and pH stability. A total of 31 lytic *E.coli* O177-specific bacteriophages were isolated. Phage isolates produced clear plaques with different sizes (diameter 1.0 ±0.02 to 2.0 ±0.05) on plaque assay. Based on different plaque morphology and clarity and size of plaques, eight phages were selected for further analyses. The spot test revealed that all selected phages were capable of infecting different environmental *E. coli* strains. However, none of the phages infected ATCC and environmental *Salmonella* strains. Furthermore, EOP analysis (range: 0.1 to 1.0) showed

that phages were capable of infecting a wide range of *E. coli* isolates. Selected phage isolates had similar morphotype (an icosahedral head and a contractile tail) and were classified under the order Caudovirales, *Myoviridae* family. The icosahedral heads ranged from 81.2 nm to 110.77 nm while the contractile tails ranged from 115.55 nm to 132.57 nm in size. The phages were found to be still active after 60 minutes of incubation at 37 °C and 40 °C. Incremental levels of pH induced a quadratic response on stability of all phages. The pH optima for all eight phages ranged between 7.6 and 8.0 while at pH 3.0 all phages were inactive. Phage latent period ranged between 15 and 25 minutes while burst size ranged from 91 to 522 virion particles (PFU) per infected cell. These results demonstrate that lytic *E. coli* O177-specific bacteriophages isolated from cattle faeces are highly stable and have the capacity to infect different *E. coli* strains, traits that make them potential biocontrol agents.

***Keywords*:** Atypical enteropathogenic *E. coli* O177; bacteriophages; biocontrol; cattle.

5.1. Introduction

Bacteriophages (phages) are self-replicating viruses, which are capable of infecting and lysing their specific host bacteria (Akhtar *et al*., 2014). They are ubiquitous replicating entities on the biosphere with an estimated number of 10^{32} (Tan *et al.*, 2014; Huang *et al.*, 2018). About ten different bacteriophages exist for each bacteria and more than 5100 phages have been identified (Harada *et al.*, 2018). Phages are relatively safe, non-toxic and harmless to animals, plants and humans (Sillankorva *et al.*, 2012; Harada *et al.*, 2018; Tang *et al.*, 2019). They are found in various environments related to their host such as in food, soil, sewage water, faeces and farm environments (Wang *et al.*, 2015; Mahmoud *et al.*, 2017; Wang *et al.*, 2017; Huang *et al.*, 2018). Several bacterial species such as *Campylobacter*, *E. coli*, *Listeria*, *Salmonella*, *Pseudomonas* and *Vibrio* species are used as hosts to isolate their specific bacteriophages (Niu

et al., 2009; Akhtar *et al.*, 2017; Mahmoud *et al.*, 2017; Tang *et al.*, 2019; Yin *et al.*, 2019; Yuan *et al.*, 2019). Because of their host specificity and non-toxicity, lytic phages are considered to be an alternative solution to combat antimicrobial resistant pathogens. Outbreak of *Listeria* and widespread occurrence of multi-drug resistance in *E. coli*, *Salmonella* and *Staphylococcus* species have been reported in South Africa (Ateba and Bezuidenhout, 2008; Akindolire *et al.*, 2015; Dlamini *et al.*, 2018; WHO, 2018). However, there has been no attempt to use bacteriophages to control antibiotic resistant pathogens, in either hospital settings or food industry.

Antibiotic resistance in foodborne pathogens, particularly *E. coli* species, remains a public health concern. Antibiotic resistant pathogens do not only increase economic and social costs but they are also responsible for severe infections in humans (Barilli *et al.*, 2019). In 2014, foodborne infections caused an estimated 600 million illnesses and 420 000 deaths across the globe (WHO, 2015). In addition, 978 listeriosis cases were reported in South Africa from 2017 to 2018, resulting in 183 deaths (WHO, 2018). The leading pathogenic bacteria of concern are *E. coli*, *Campylobacter*, *Listeria* and *Salmonella* species (Farrokh *et al.*, 2013). In addition, recent reports revealed that non-O157 strains, particularly O26, O45, O103, O111, O121 and O145, exhibit multi-drug resistance and are among the leading causes of foodborne infection (Gould *et al.*, 2013; Iwu *et al.*, 2016; Ntuli *et al.*, 2017).

Cattle are known to be the primary reservoirs of antibiotic resistant *E. coli* strains (Farrokh *et al.*, 2013). Furthermore, indiscriminate use of antibiotics in food-producing animals may result in extensive spread of antibiotic resistance determinants between the same and/or different bacteria species (Koo and Woo, 2011; Qiao *et al.*, 2017). In addition, *E. coli* species harbour multi-drug resistance plasmids, which may accelerate the transfer of resistance genes to other

transient bacterial pathogens. Against this background, several studies have investigated and reported the occurrence of antibiotic resistance genes in *E. coli* isolated from different sources, including cattle faeces (Ateba and Bezuidenhout, 2008; Ahmed and Shimamoto, 2015; Bergeron *et al.*, 2015; Abia *et al.*, 2016; Qiao *et al.*, 2017). Given that carcasses are vulnerable to faecal contamination during slaughtering process, antibiotic resistant atypical enteropathogenic *E. coli* O177 strain and other foodborne pathogens may be transferred to humans through consumption of contaminated food.

In view of the above, several interventions, such as physical, chemical and biological methods, have been devised and implemented at all levels of food chain to combat foodborne infection and the spread of antibiotic resistant pathogens (Hungaro *et al.*, 2013; Kazi and Annapure, 2016). However, these conventional methods have significant drawbacks such as corrosion of food processing plants, environmental pollution, change of food matrices, development of antibiotic resistance and toxic effects of chemical residues (Chen *et al.*, 2012). In addition, application of chemical agents in food may hamper international trade and affect the economy of the exporting country (Hungaro *et al.*, 2013). Moreover, the continued rise in foodborne infections due to consumption of contaminated food clearly demonstrates that these strategies are not infallible (Kazi and Annapure, 2016). Furthermore, the use of probiotics as biological intervention strategy has its own limitations (Harada *et al.*, 2018). For example, some probiotics may work efficiently in simple non-ruminants than in ruminants. Furthermore, probiotics may elicit changes in the gut microbiota and thus affect diet digestibility. The lack of new antibiotics and inefficacy of conventional strategies to combat multi-drug resistant bacterial pathogens necessitates the search for alternative control strategies such as the use of bacteriophages.

Considering the virulence and antibiotic resistance profiles of *E. coli* O177 strain, coupled with lack of new antibiotics and limitations of conventional strategies to mitigate antibiotic resistance, there is a need to expand the search for novel bacteriophages capable of curbing sporadic outbreaks of diseases caused by antibiotic resistant pathogens. Therefore, the current study was designed to isolate and characterise lytic *E. coli* O177-specific bacteriophages as potential biocontrol agents. Stability and viability of the phages was determined under temperature and pH ranges to assess their stability for pre-harvest use in these animals.

5.2. Materials and methods

5.2.1. Bacteria strain

Multi-drug resistant and virulent atypical enteropathogenic *E. coli* O177 strain was used to isolate *E. coli* O177-specific bacteriophages. The atypical enteropathogenic *E. coli* O177 isolates were obtained from cattle faeces and the isolates were confirmed through PCR analysis. The isolates were further screened for the presence of virulence and antimicrobial gene determinants. Prior to phages isolation, forty *E. coli* O177 isolates stored at −80 °C were resuscitated on MacConkey agar and incubated at 37 °C for 24 hours. A single colony from each sample was transferred into 15 mL nutrient broth in 50 mL falcon tubes. The samples were incubated in a shaker (160 rpm) at 37 °C for three hours until the growth reached an optical density (OD) of 0.4 to 0.5 (600 nm).

5.2.2. Enrichment and isolation of *E. coli* O177–specific bacteriophages

Escherichia coli O177-specific bacteriophages were isolated from cattle faeces using environmental *E. coli* O177 strain following the enrichment method (Sambrook *et al.*, 1989; Van Twest and Kropinski, 2009) with some modifications. Twenty faecal samples were collected from two commercial feedlots and two dairy farms. Samples were collected directly

from the rectum using arm-length rectal gloves, placed in cooler box containing ice packs and transported to the laboratory. Five grams of each faecal sample were dissolved in 20 mL lambda diluent and vortexed to obtain a homogeneous mixture. The mixture was centrifuged at 10 000 × g for 10 minutes using Hi Centrifuge SR (Model: Z300, Germany) to sediment faecal matter and other impurities. An aliquot of 10 mL from the supernatant was extracted and filter-sterilised using 0.22 µm pore syringe filter (GVS Filter Technology, USA) to obtain crude phage filtrates. The enrichment process was carried out by adding 5 mL of each filtrate to 100 µL of exponential culture of *E. coli* O177 and the mixture was transferred into 50 mL falcon tube containing 10 mL double-strength tryptic soya broth (TSB) supplemented with 2 mM calcium chloride ($CaCl_2$). The samples were incubated in a shaking incubator (80 rpm) at 37 °C for 24 hours. After incubation, the samples were centrifuged at 10 000 × g for 10 minutes using Hi Centrifuge SR (Model: Z300, Germany) to remove bacterial cells and sample debris. The supernatant was filter-sterilised with 0.22 µm pore-size acrodisc syringe filter (GVS Filter Technology, USA) to obtain crude phage filtrates.

Subsequently, spot test was performed to determine the presence of phages as previously described (Sambrook *et al.*, 1989). Briefly, 100 µL of exponential phase (OD_{600} = 0.4 to 0.5) culture of the bacterial host was mixed with 3 mL of soft agar (0.3% w/v) held at 50 ºC and then poured onto modified nutrient agar (MNA) plates so as to create a bacterial lawn and allowed to solidify for 15 minutes. Ten microliters of each crude phage filtrate was spotted on bacterial lawn and the plates were incubated at 37 °C for 24 hours. After incubation, the plates were observed for the presence of clear zones or plaques at inoculated points. Plaques were picked using a sterile pipette tip and suspended in 1 mL lambda diluent [10 mM Tris Cl (pH 7.5), 8 mM $MgSO_4.7H_2O$] in 2 µL eppendorf tubes. The tubes were left at room temperature for 18 hours to allow phage to diffuse into the solution. The tubes were then centrifuged at

11000 × g for 10 minutes and the supernatant was filter-sterilised with 0.22 µm pore-size acrodisc syringe filter (GVS Filter Technology, Germany).

Plaque assay was performed to isolate and purify phages as previously described (Sambrook and Russell, 2001). Briefly, ten-fold serial dilutions of crude phage lysates were prepared and aliquot of 100 µL of each phage lysate was mixed with 100 µL of exponential phase (OD_{600} = 0.4 to 0.5) culture of corresponding host(s) in 50 mL sterile falcon tubes and left for 10 minutes at the room temperature to allow the phage to attach to the host. Then, 3 mL of soft agar (0.3% w/v) was added to the tube and the mixture was poured onto MNA plates. The plates were allowed to solidify and incubated at 37 °C for 24 hours. After incubation, the plates were checked for the presence of plaques. If present, plaques from each plate were picked based on their sizes and clarity using a sterile pipette tip and were resuspended in 1 mL lambda diluent in 2 µL eppendorf tubes. The tubes were left at 4 °C for 24 hours to allow phage to diffuse into the solution. The tubes were then centrifuged at 10 000 × g for 10 minutes and the supernatant was filter-sterilised using 0.22 µm pore-size acrodisc syringe filter (GVS Filter Technology, Germany). Purification process was repeated three consecutive times until homogeneous plaques were obtained for each phage isolate.

5.2.3. Propagation and titration of *E. coli* O177-specific bacteriophages

Purified phages were propagated using *E. coli* O177 host bacteria. One hundred microliters of pure phage stocks were mixed with 100 µL of exponential phase (OD_{600} = 0.4 to 0.5) culture of corresponding host(s) in 50 mL falcon tube containing sterile 10 mL double-strength tryptic soya broth (TSB) supplemented with 2 mM $CaCl_2$. The mixture was incubated in a shaking incubator (150 rpm) at 37 °C for 24 hours. After incubation, the samples were centrifuged at 8000 × g for 10 minutes at 4 °C and the supernatant was filter-sterilised using 0.22 µm pore-

size acrodisc syringe filter (GVS Filter Technology, Germany). Ten-fold serial dilutions were prepared, phage titers were determined using plaque assay and the titers were expressed as plaque-forming unit per milliliter (PFU/mL). The stock phages were stored at 4 °C for further analysis.

5.2.4. Phage host range determination and efficiency of plating analysis

5.2.4.1. Determination of host range and cross infectivity of the phage isolates

The host range of eight selected phage isolates was evaluated against 50 bacterial hosts [13 *E. coli* O177, 12 *E. coli* O157, 12 *E. coli* O26 and 10 *Salmonella* species (environmental strains), 1 *Pseudomonas aeruginosa* (ATCC 27853) and 1 *S. enterica* (ATCC 12325) and 1 Salmonella Typhimurium (ATCC 14028)] and all environmental species were isolated from cattle faeces. Phage isolates were selected based on different plaque morphologies; clarity of the plaques and sizes. The spot test technique was performed to determine lytic spectrum activity of each phage isolates as previously described (Adams, 1959). The bacteria lawns of all the selected bacteria hosts were prepared on MNA plates. Ten microliters of phage stock (10^7 to 10^9 PFU/mL) was spotted on bacterial lawn and allowed to air-dry under laminar airflow for 10 minutes. The plates were incubated at 37 °C for 24 hours. After incubation, the plates were observed for the presence of plaques at the point of application and the phage lytic profiles were classified into three categories according to their clarity: clear, turbid and no lysis (Zhang *et al.*, 2018). The test was performed in triplicates for each phage isolate.

5.2.4.2. Efficiency of plating of phages

Efficiency of plating (EOP) was performed to determine lytic efficiency of the phage in comparison with their suitable host bacteria as previously described (Kutter, 2009), with modification. Fifteen bacterial strains (5 *E. coli* O177, 5 *E. coli* O26 and 5 *E. coli* O157) were

selected based on their sensitivity against the phages. *Escherichia coli* O177 isolates (CF-D-D, CF-A27, CF-H361, CF-A28 and CF-D-D246 were used as references). Ten-fold serial dilutions of phage were prepared to obtain single plaques. Aliquot of 100 µL of each phage (1 × 10^4 PFU/mL) was mixed with 100 µL of exponential phase (OD_{600} = 0.4 to 0.5) culture of each bacteria in 50 mL sterile falcon tubes and left for 10 minutes at the room temperature to allow the phage to attach to the host. Then, 3 mL of soft agar (0.3% w/v) was added to the tube and the mixture was poured onto MNA plates. Three independent assays were performed for each phage isolate. After solidifying, the plates were incubated at 37 °C for 24 hours. After incubation, the number of plaques per plate were counted. The EOP was calculated as the ratio between the average number of plaques on target host bacteria (PFUs) and average number of plaques of reference host bacteria (PFUs). The EOP were classified as high (EOP ≥ 0.5), moderate (EOP > 0.1 - < 0.5) and low (EOP ≤ 0.1) based on the reproducible infection on the targeted bacteria (Manohar *et al.*, 2019). The following formula was used to calculate EOP values:

$$\text{Relative EOP} = \frac{\text{average number of plaques on targeted host bacteria (PFUs)}}{\text{average number of plaques on reference host bacteria (PFUs)}}$$

5.2.5. Polyethylene glycol precipitation

Prior to transmission electron microscopy, phages were concentrated with 10% (w/v) polyethylene glycol (PEG, MW 8000) precipitation method (Sambrook *et al.*, 1989). Briefly, phages were propagated to obtain high titer (10^8 to 10^{11} PFU/mL). Ten milliliters of each phage (10^8 to 10^{11} PFU/mL) were concentrated in 50 mL falcon tubes by adding 10% (w/v) PEG and the mixture was incubated at 4 °C overnight to allow precipitation of the phage particles. The following day, phage particles were sedimented by centrifugation at 11 000 × g for 10 minutes at 4 °C. The supernatant was discarded and the pellet was washed three times with 0.1 M ammonium acetate (pH 7.0). The pellet was resuspended in 200 µL of ammonium acetate.

Samples were transported in a cooler box containing ice parks to the Virology Laboratory at the North-West University, Potchefstroom Campus, for transmission electron microscopy.

5.2.6. Transmission electron microscopy (TEM) analysis

Eight phage isolates were subjected to transmission electron microscopy and phage morphotype was determined using negative staining techniques as previously described (Brenner and Horne, 1959), with some modifications. Briefly, a drop of concentrated phage solution was deposited on 200 mesh copper grids with carbon-coat formvar films. The phage particles were allowed to adsorb for 2 minute and excess liquid was drained off with a sterile filter paper. The grid was allowed to air dry. A drop of 1% (w/v) ammonium molybdate (aqueous, pH 6.5) was added to negatively stain the phage particles and allowed to air dry for 10-15 minutes. The grid containing the specimen (phage particles) was then loaded into transmission electron microscope (Model: FEI TECHNAI; TEM, Czech Republic) and operated at 120 KV to scan and view phage images with magnification range of 20000 – 100000. Micrographs were taken with a Gatan bottom mount camera using Digital Micrograph software at 80 kV and a magnification range of 20,000 to 250,000. The images were taken and morphology characteristics were used to classify phage isolates as previously described (Ackermann, 2009).

5.2.7. Effect of different temperatures on the stability and viability of phages

Phage stability and viability were evaluated across different temperatures (37 °C and 40 °C) over a 60-minute period in a temperature-controlled incubator. The concentration of the host bacteria and phage titers were standardised before starting the experiment. One hundred microliters of exponential phase culture (10^5 CFU/mL) and 100 µL of phage (10^5 PFU/mL) were added to 10 mL double strength TSB supplemented with 2 mM $CaCl_2$. The tubes were

incubated in a pre-set shaking incubator at 37 °C and 40 °C for 60 minutes and samples were taken at 10, 30 and 60 minutes of incubation and assessed for viability and concentration using double layer agar (Sambrook and Russell, 2001). Plaque assay was performed in triplicates for each sample and the results were expressed as plaque forming unit per millilitre (PFU/mL).

5.2.8. Effect of different pH levels on the stability and viability of phages

Phage stability and viability were evaluated at different pH levels (3.0, 4.5, 6.3, 7.0, 8.5, and 10.0) in a 48-hour incubation period. Both phage and bacterial host were prepared following the same procedure described in Section 5.2.7 above. Ten milliliters of sterile double strength TSB (amended with 2 mM $CaCl_2$) was distributed into 50 mL falcon tubes to prepare different pH solutions. The pH (7.2) of the TSB solutions was adjusted using hydrochloric acid (HCL, 6 M) or sodium hydroxide (NaOH, 6 M) to obtain the desired pH levels (3.0, 4.5, 6.3, 7.0, 8.5, and 10.0). One hundred microliters of each bacterial host (10^5 CFU/mL) and their corresponding phage (10^5 PFU/mL) isolates were added to 10 mL. The tubes were incubated in a pre-set shaking incubator (80 rpm) at 37 °C for 48 hours. Samples were taken at 24 and 48 hours incubation and phage titre for each sample was determined using standard plaque assay as previously described (Sambrook and Russell, 2001). Plaque assay was performed in triplicates for each sample and the results were expressed as plaque forming unit per milliliter.

5.2.9. Determination of phage growth parameters

The life cycle of phage isolates was evaluated *in vitro* using the one step growth procedure as previously described (Adams, 1959), with some modifications. Briefly, 5 mL of exponential phase culture of each host was centrifuged at 8000 × g for 5 minutes at 4 °C. The pallet was resuspended in 10 mL double strength TSB supplemented with 2 mM $CaCl_2$ to obtain OD of 0.4 to 0.5 (600 nm). Aliquot of 100 was removed and ten-fold serial dilution was prepared and

plated on nutrient agar to determine bacterial concentration (CFU/mL). One hundred microliters of each phage isolates (10^8 PFU/mL) were added to their respective host bacteria suspension to achieve multiplicity of infection (MOI) of 1.0. The mixture was left at room temperature for 10 minutes to allow phages to adsorb to the host bacteria. After 10 minutes, 1.5 µL of the mixture was transferred into 2 µL eppendorf tubes and centrifuged at 11000 × g for 10 minutes to remove unadsorbed phage particles. The pellet was resuspended in 100 µL of TSB supplemented with 10 mM magnesium sulphate (mTSB) and transferred into a pre-warmed 9.9 mL mTSB. The samples were incubated in shaking incubator (160 rpm) at 37 °C for 1 hour. Two hundred microliter was drawn from each sample at 5-minute intervals for 60 minutes. Plaque assay was performed in triplicates for each samples to determine phage titer. The data generated was used to determine latent period, burst time and phage relative burst size per infected cell. The burst size was calculated as the ratio of the final count of released phage progeny to the initial count of infected bacteria host cell during the latent period using the following formula as previously described (El-Dougdoug *et al.*, 2019):

$$\text{Relative burst size} = \frac{\text{final titer (pfu)} - \text{initial titer (pfu)}}{\text{initial titer (pfu)}}$$

The relative burst size at different time point was plotted against time to determine the latent period and burst size of each phage isolate.

5.2.10. Statistical analysis

Measured parameters were tested for normality using the NORMAL option in the Proc Univariate statement as well as for homogeneity of variances using the Levene's test option in the Means statement of the GLM procedure of SAS prior to analysis of variance. The viability and stability of phages were tested at different temperatures and pH levels. The data were

converted to $\log_{10}$ PFU/mL and analysed using SAS (2010). The effect of temperature, time and phage type on viability and stability of phages was analysed using the general linear models (GLM) procedure of SAS (2010) for a 2 (temperature) × 3 (time) × 8 (phages) factorial treatment arrangement according to the following model:

$$Y_{ijkl} = \mu + T_i + S_j + V_k + (T\times S)_{ij} + (T\times V)_{ik} + (S\times V)_{jk} + (T\times S\times V)_{ijk} + E_{ijkl},$$

where Y_{ijkl} is the observation of the dependent variable $_{ijkl}$; μ is the fixed effect of population mean for the variable; T_i is the effect of temperature; S_j is the effect of time; V_k is the effect of phages; $(T\times S)_{ij}$ is the effect of interaction between temperature at level *i* and time at level *j*; $(T\times V)_{ik}$ is the effect of interaction between temperature at level i and phages at level k; $(S\times V)_{jk}$ is the effect of interaction between time at level *j* and phage at level *k*; $(T\times S\times V)_{ijk}$ is the effect of interaction between temperature at level *i*, time at level *j* and phage at level *k*, and E_{ijkl} is the random error associated with observation *ijkl*.

Phage viability and stability data in response to incremental levels of pH were evaluated for linear and quadratic effects using polynomial contrasts. Response surface regression analysis (Proc RSREG; SAS 2010) was applied to describe the responses to pH according to the following quadratic model: $y = a + bx + cx^2$, where y = response variables, b and c are the coefficients of the quadratic equation; a is intercept; *x* is pH level and $-b/2c$ is the *x* value for maximum response. For all statistical tests, significance was declared at $P \leq 0.05$.

5.3. Results

5.3.1. Isolation, purification and propagation of bacteriophages

Thirty-one lytic *E. coli* O177-specific bacteriophages were isolated from cattle faeces. Phage isolates were as assigned vB_EcoM_number as their identity. The vB_EcoM_number denotes **v**irus of **b**acteria infecting ***E****scherichia* ***co****li*, with **m**yovirus morphology and the sample **n**umber. Plaque morphology of the phage isolates is depicted in Table 5.1 and Figure 5.1 below. Phage isolates revealed different plaque morphology in terms of sizes, ranging from small (1 mm) to large (2 mm) plaques, Table 5.1 and Figure 5.1. Large proportion (71%) of the phage isolates revealed large plaques while a small proportion (29%) showed small plaques on *E. coli* O177 strain. As indicated in Table 5.1 and Figure 5.1, all the phages revealed clear (complete lysis) plaques, indicating that they are lytic and none of the phage isolates revealed turbid plaques. Phage titer after propagation ranged from 6.2×10^5 to 3.1×10^{13} PFU/mL. Phage vB_EcoM_3A2 had the lowest titre while phage vB_EcoM_198B1 had the highest titer compared to other phage isolates.

Table 5.1: Plaque morphology of 31 *E. coli* O177-specific bacteriophages isolated from cattle faeces.

Bacterial Host			Phages								
								Plaque morphology			
								Lysis			Size
Bacteria ID	**Dilution Vector**	**Volume***	**Phage (ID)**	**Dilution Vector**	**Volume***	**No. plaques**	**PFU/mL**	**clear**	**turbid**	**no lysis**	
CF-D-D202	10^{-5}	100	vB_EcoM_3B2	10^{-6}	100	206	2.06E+09	+++	-	-	2 mm[a]
CF-D-D202	10^{-5}	100	vB_EcoM_3C2	10^{-5}	100	85	8.50E+07	+++	-	-	2 mm[a]
CF-A28	10^{-6}	100	vB_EcoM_366V	10^{-7}	100	39	3.90E+09	+++	-	-	2 mm[a]
CF-A27	10^{-7}	100	vB_EcoM_12B2	10^{-7}	100	65	6.50E+09	+++	-	-	1 mm[b]
CF-A5	10^{-6}	100	vB_EcoM_198A1	10^{-5}	100	35	3.50E+07	+++	-	-	2 mm[a]
CF-A5	10^{-6}	100	vB_EcoM_198B2	10^{-4}	100	70	7.00E+06	+++	-	-	2 mm[a]
CF-H 361	10^{-6}	100	vB_EcoM_10C1	10^{-6}	100	90	9.00E+08	+++	-	-	1 mm[b]
CF-D-D202	10^{-5}	100	vB_EcoM_3C1	10^{-6}	100	42	4.20E+08	+++	-	-	2 mm[a]
CF-D-D202	10^{-5}	100	vB_EcoM_3A3	10^{-3}	100	165	1.65E+06	+++	-	-	2 mm[a]
CF-D-D202	10^{-5}	100	vB_EcoM_3A2	10^{-3}	100	62	6.20E+05	+++	-	-	2 mm[a]

CF-D-D202	10^{-5}	100	vB_EcoM_3A1	10^{-9}	100	129	1.29E+12	+++	-	-	2 mm[a]
CF-H361	10^{-6}	100	vB_EcoM_10B1	10^{-8}	100	37	3.70E+10	+++	-	-	2 mm[a]
CF-A27	10^{-7}	100	E vB_EcoM_12A2	10^{-6}	100	33	3.30E+08	+++	-	-	1 mm[b]
CF-H361	10^{-6}	100	vB_EcoM_10C2	10^{-8}	100	111	1.11E+11	+++	-	-	2 mm[a]
CF-A5	10^{-6}	100	vB_EcoM_10B2	10^{-4}	100	112	1.12E+07	+++	-	-	1 mm[b]
CF-A5	10^{-6}	100	vB_EcoM_198A2	10^{-4}	100	35	3.50E+06	+++	-	-	2 mm[a]
CF-D-D202	10^{-5}	100	vB_EcoM_3B4	10^{-4}	100	120	1.20E+07	+++	-	-	2 mm[a]
CF-D-D202	10^{-5}	100	vB_EcoM_3B3	10^{-4}	100	70	7.00E+06	+++	-	-	2 mm[a]
CF-D-D202	10^{-5}	100	vB_EcoM_3B1	10^{-7}	100	30	3.00E+09	+++	-	-	2 mm[a]
CF-D-D246	10^{-6}	100	vB_EcoM_118A	10^{-6}	100	92	9.20E+08	+++	-	-	1 mm[b]
CF-D-D246	10^{-6}	100	vB_EcoM_118B	10^{-9}	100	151	1.51E+12	+++	-	-	2 mm[a]
CF-A27	10^{-7}	100	vB_EcoM_12A1	10^{-8}	100	145	1.45E+11	+++	-	-	2 mm[a]
CF-A27	10^{-6}	100	vB_EcoM_12B1	10^{-3}	100	155	1.55E+06	+++	-	-	1 mm[b]
CF-A5	10^{-6}	100	vB_EcoM_198A3	10^{-7}	100	67	6.70E+09	+++	-	-	1 mm[b]
CF-A28	10^{-6}	100	vB_EcoM_11B1	10^{-7}	100	38	3.80E+09	+++	-	-	1 mm[b]

CF-A28	10^{-6}	100	vB_EcoM_11A1	10^{-7}	100	35	3.50E+09	+++	-	-	1 mm[b]
CF-D-D246	10^{-6}	100	vB_EcoM_366B	10^{-10}	100	40	4.00E+12	+++	-	-	2 mm[a]
CF-D-D246	10^{-6}	100	vB_EcoM_366A	10^{-4}	100	30	4.00E+05	+++	-	-	2 mm[a]
CF-A5	10^{-6}	100	vB_EcoM_198B1	10^{-11}	100	31	3.10E+13	+++	-	-	2 mm[a]
CF-A28	10^{-6}	100	vB_EcoM_11B2	10^{-8}	100	110	1.10E+11	+++	-	-	2 mm[a]
CF-H361	10^{-6}	100	vB_EcoM_10C3	10^{-9}	100	97	9.70E+11	+++	-	-	2 mm[a]

***Key*:** ID = identity; Volume* = Inoculum size (µL); **+++ =** complete clear zone; **- =** no lysis; superscripts "**a** and **b**" denotes large and small, respectively.

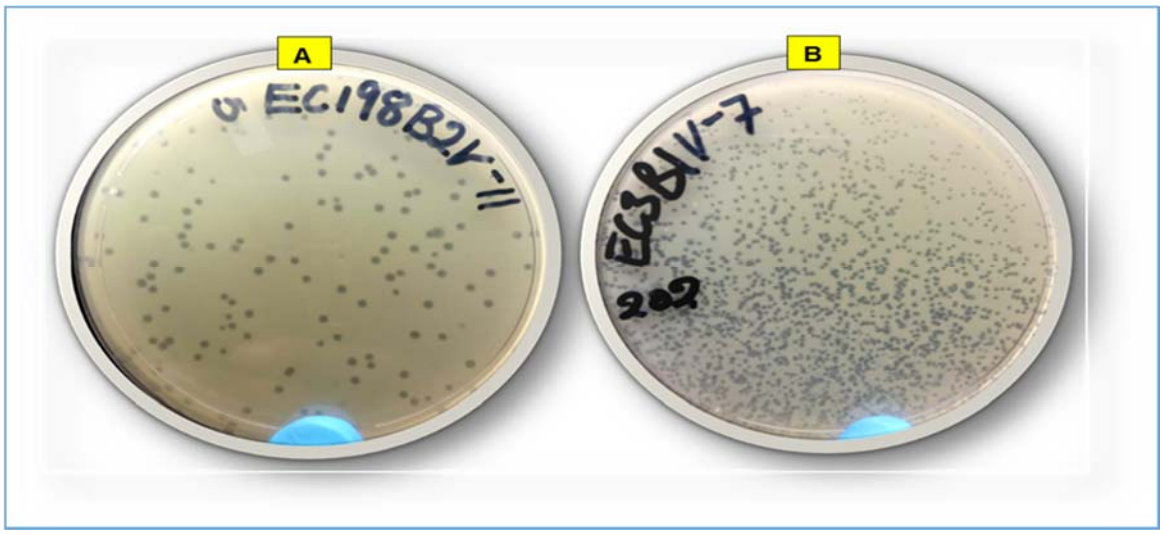


Figure 5.1: Representative image of phage isolates depicting different plaque morphology, **A** = vB_EcoM_198B2 (large plaques), **B** = vB_EcoM_3B1 (small plaques).

5.3.2. Host range of phages and EOP analysis against different *E. coli* strains

Spot test was performed to determine the host range of eight selected lytic phage isolates against 50 bacteria hosts comprising of different bacterial species. The results indicated that the phages were capable of infecting *E. coli* species (*E. coli* O177, *E. coli* 0157 and *E. coli* O26 environmental strains) tested, Figure 5.2. All phage isolates produced clear plaques against all *E. coli* O177 and 83- 100% of *E. coli* O26 serotypes, Table 5.2. Three phages (vB_EcoM_10C3, vB_EcoM_11B2 and vB_EcoM_12A1) were able to infect *E. coli* O157 (75- 83%), Table 5.2. None of the phages could infect ATCC strains and environmental *Salmonella* species. The EOP analysis was performed on 15 (5 *E. coli* O177, 5 *E. coli* O26 and 5 *E. coli* O157) isolates that were susceptible to phages on spot test. Although spot test results revealed clear plaques on *E. coli* O177, EOP results exhibited various lytic patterns of the phages. Even though EOP analysis revealed high (EOP ≥ 0.5) productive infection on *E. coli* O177, moderated infections were observed, Table 5.3. Four phages revealed high EOP values (0.5 to 0.8) on *E. coli* O177 isolates. On the other hand, EOP analysis exhibited moderate and

low productive infection on *E. coli* O26 and *E. coli* O157 isolates (EOP values range from 0.0 to 0.4 and 0.0 to 0.3, respectively).

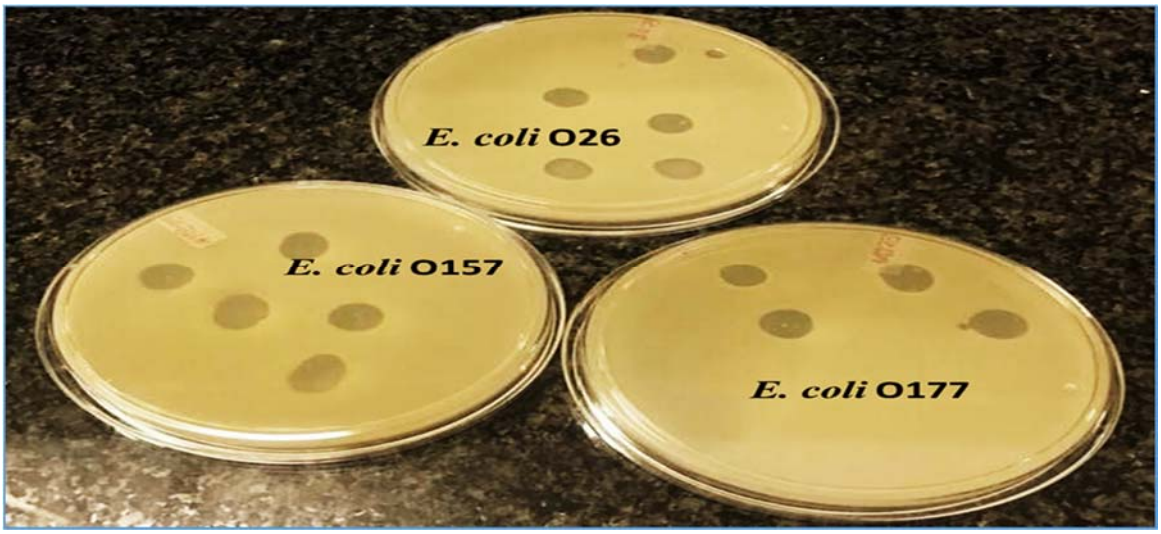


Figure 5.2: Representative image depicting spot test results of phages on different *E. coli* strains.

Table 5.2: Host range analysis of *E. coli* O177-specific phages.

Host Bacteria	No	Phage host range (%)							
		vB_EcoM_10C2	vB_EcoM_10C3	vB_EcoM_118B	vB_EcoM_11B2	vB_EcoM_12A1	vB_EcoM_366B	vB_EcoM_366V	vB_EcoM_3A1
***Pseudomonas aeruginosa*[a]**	1	1(0%)	1(0%)	1(0%)	1(0%)	1(0%)	1(0%)	1(0%)	1(0%)
***S. enterica*[b]**	1	1(0%)	1(0%)	1(0%)	1(0%)	1(0%)	1(0%)	1(0%)	1(0%)
***S. typhimurium*[c]**	1	1(0%)	1(0%)	1(0%)	1(0%)	1(0%)	1(0%)	1(0%)	1(0%)
***E. coli* O177[d]**	13	13(100%)	13(100%)	13(100%)	13(100%)	13(100%)	13(100%)	13(100%)	13(100%)
***E.coli* O26[d]**	12	12(100%)	10(83%)	11(92%)	11(92%)	10(83%)	11(92%)	11(92%)	11(92%)
***E. coli* O157[d]**	12	12(0%)	9(75%)	12(0%)	10(83%)	12(0%)	12(0%)	12(0%)	10(8%)
***Salmonella* species[d]**	10	10(0%)	10(0%)	10(0%)	10(0%)	10(0%)	10(0%)	10(0%)	10(0%)

***Key*:** Superscripts "**a**, **b**, c and **d**" denote ATCC 27853; ATCC 12325; ATCC 14028 and environmental strains, respectively.

Table 5.3: Efficacy of plating (EOP) of phages against different *E. coli* serotypes.

Bacteria serotype	Bacteria ID	EOP ratio of phage isolates							
		vB_EcoM_10C2	**vB_EcoM_10C3**	**vB_EcoM_118B**	**vB_EcoM_11B2**	**vB_EcoM_12A1**	**vB_EcoM_366B**	**vB_EcoM_366V**	**vB_EcoM_3A1**
E. coli O177	CF-D-D202	0.7	0.8	0.6	0.5	0.7	0.8	0.8	1.0[a]
	CF-A27	0.5	0.6	0.7	0.7	1.0[a]	0.8	0.7	0.6
	CF-H361	1.0[a]	1.0[a]	0.5	0.6	0.8	0.8	0.6	0.7
	CF-A28	0.4	0.7	0.5	1.0[a]	0.5	0.5	1.0[a]	0.8
	CF-D-D246	0.7	0.9	1.0[a]	0.8	0.8	1.0	0.8	0.7
E. coli O26	2A	0.3	0.2	0.4	0.1	0.0	0.4	0.2	0.0
	4C	0.3	0.2	0.2	0.1	0.1	0.1	0.3	0.1
	17E	0.2	0.2	0.1	0.1	0.3	0.3	0.2	0.3
	21F	0.1	0.1	0.3	0.3	0.2	0.2	0.1	0.1
	25H	0.3	0.3	0.1	0.3	0.1	0.3	0.2	0.1
E. coli O157	1A	0.1	0.1	0.2	0.1	0.0	0.1	0.3	0.3
	3B	0.2	0.2	0.1	0.1	0.0	0.1	0.1	0.1
	5D	0.3	0.1	0.1	0.2	0.1	0.1	0.1	0.2
	7F	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1
	8G	0.1	0.3	0.1	0.3	0.1	0.2	0.1	0.1

***Key*:** ID = identity; superscript "**a**" denotes reference host.

5.3.3. Morphological characterisation of phages based on transmission electron microscopy

Eight selected phage isolates were subjected to transmission electron microscopy (TEM) analysis to determine their morphotype. Transmission electron micrographs images of the phages and structural dimensions are shown in Figure 5.3 and Table 5.4, respectively. Phage isolates were classified as per the International Committee on Taxonomy of Virus (ICTV) classification based on three-dimensional structure observed. All phage isolates showed similar morphotype on TEM analysis. Structurally, the phages had icosahedral head, neck attached to a long contractile tail, with tail fibres and they were classified under the order Caudovirales, *Myoviridae* family. The phage icosahedral heads ranged from 81.2±6 nm to 95.6±3 nm while the contractile tails ranged from 118.1±0.3 nm to 135±2 nm. Phage vB_EcoM_10C2 had a longest icosahedral head with diameter of 95.6±3 nm and the longest contractile tail of 135±2 nm with fibres. Phage vB_EcoM_10C3 had the smallest icosahedral head of 81.2±6 nm diameter and the shortest contractile tail of 118.1±0.3 nm with fibres.

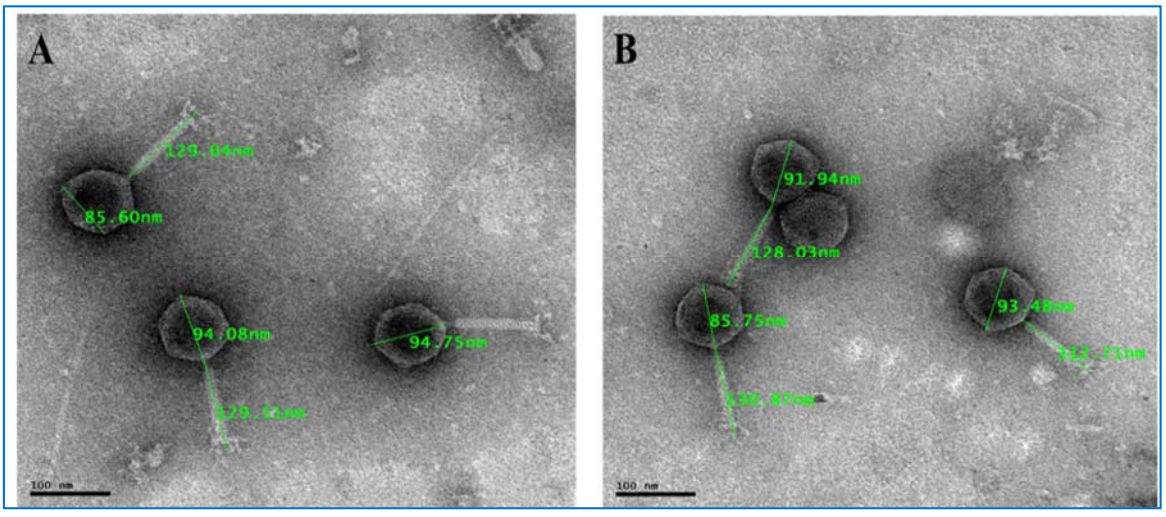


Figure 5.3. Transmission electron micrographs images of representative phage isolates (negatively stained with 1% ammonium molybdate). Both phages (A = vB_EcoM_11B2 and B = vB_EcoM_118B) belong to myoviridae family and are showing icosahedral capsid and long contractile tail with tail fibres. The bars indicate scale (100 nm).

Table 5.4. Phage dimensions based on TEM analysis.

Phage ID	Phage morphotype				Order
	Head structure	Head dimensions (nm±stdv)	Tail structure	Tail dimensions (nm±stdv)	
vB_EcoM_10C2	icosahedral capsid	95.6±3	Contractile sheath	135±2	Caudovirales
vB_EcoM_10C3	icosahedral capsid	81.2±6	Contractile sheath	118.1±0.3	
vB_EcoM_118B	icosahedral capsid	90.4±3	Contractile sheath	123.9±6	
vB_EcoM_11B2	icosahedral capsid	91.5±3	Contractile sheath	129.3±0.2	
vB_EcoM_12A1	icosahedral capsid	87.8±2	Contractile sheath	121.9±6	
vB_EcoM_366B	icosahedral capsid	88.5±3	Contractile sheath	129.8±3	
vB_EcoM_366V	icosahedral capsid	86.7±2	Contractile sheath	120.3±9	
vB_EcoM_3A1	icosahedral capsid	85.6±1	Contractile sheath	119.3±1	

5.3.4. Phage stability and viability against different temperatures

The results showed significant ($p < 0.001$) time × temperature interaction effect on the stability and viability of the phages. Incubation of phages from 10 to 60 minutes resulted in significant phage growth at 37 °C, Figure 5.4. The growth from 10 to 30 minutes ranged from 8.55 $\log_{10}$ PFU/mL to 8.75 $\log_{10}$ PFU/mL (at 37 °C). Phage vB_EcoM_3A1 revealed the lowest growth rate while phage vB_EcoM_10C3 exhibited the fastest growth rate from 10 to 60 minutes. Phage growth at 40 °C when incubated for 10 to 60 minutes is depicted in Figure 5. 5. From 10 to 30 minutes, seven phages revealed significant growth rate (ranging from 8.71 $\log_{10}$ PFU/mL to 9.13 $\log_{10}$ PFU/mL). A decrease in phage vB_EcoM_12A1 growth rate was

observed (from 10 to 60 minutes) while two phages (vB_EcoM_10C2 and vB_EcoM_10C3) exhibited decrease in growth rate from 30 to 60-minute incubation period.

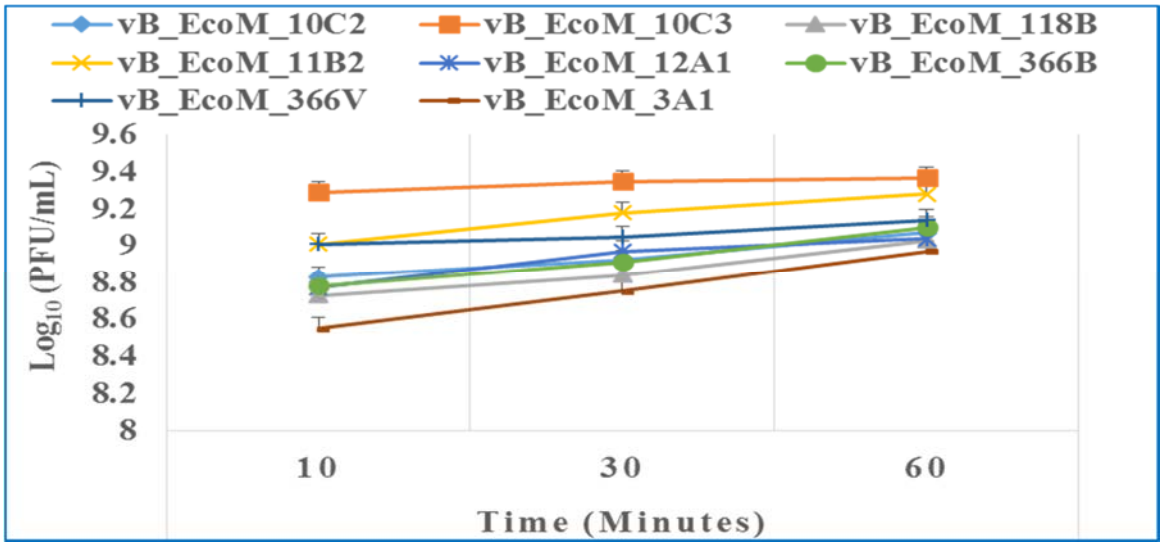


Figure 5.4: Effect of time on persistence (stability/survivability) of individual phages at 37 °C. The error bars represent the standard deviation.

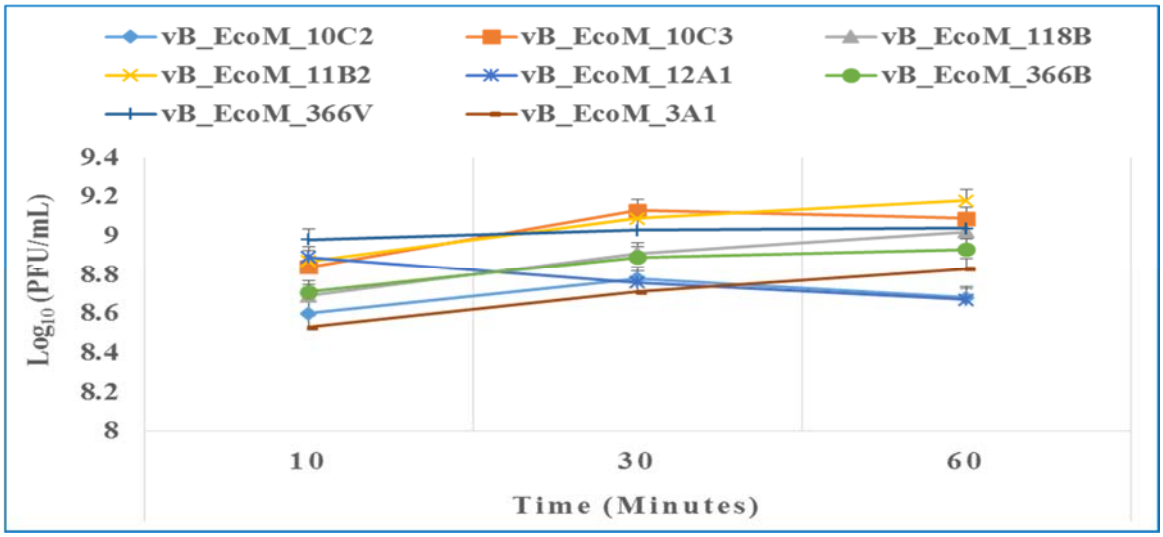


Figure 5.5: Effect of time on persistence (stability/survivability) of individual phages at 40°C. The error bars represent the standard deviation.

An exposure of individual phages to 37 °C for 10 to 30 minutes showed significant increase in growth rate with time (0.4% to 2.3% growth rate at 37 °C, respectively), Figure,5. 6 and 5.7. The average growth rate of the phages increased from 0.2 to 0.17 $\log_{10}$ PFU/mL at 37 °C (10 to 30-minute incubation period). Phages revealed various response when incubated at 40 °C. Decline in growth rate in other phages was observed. When incubated for 10 minutes at 40 °C, vB_EcoM_10C3 growth rate declined the most by 4.8% while phage vB_EcoM_3A1 growth rate was least affected showing only a 0.2% decline. After 30-minute incubation, decline in growth rate was observed in phage vB_EcoM_366B (0.2%), vB_EcoM_366V (0.2%) and vB_EcoM_10C3 indicated (2.4%), Figure 5.7. When incubated for 60 minutes at 40 °C, phages vB_EcoM_10C2 (0.28 $\log_{10}$ PFU/mL), vB_EcoM_10C3 (0.39 $\log_{10}$ PFU/mL) and vB_EcoM_12A1 (0.37 $\log_{10}$ PFU/mL) exhibited the greatest decline in growth rate when compared to their respective rate for 60 minutes at 37 °C.

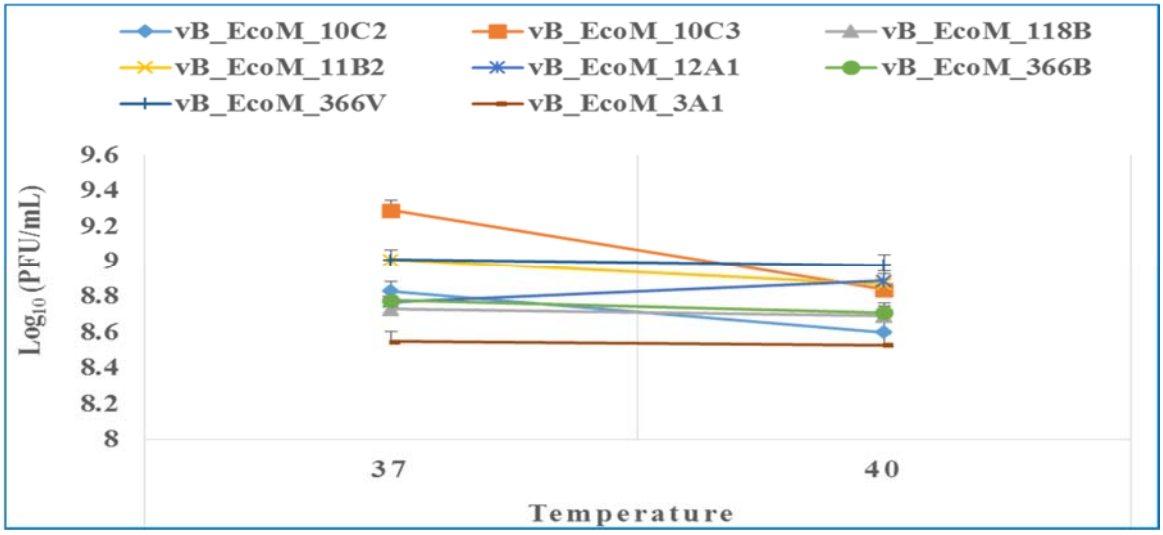


Figure 5.6: Survival and stability of individual phages when exposed to different temperatures for 10 minutes. The error bars represent the standard deviation.

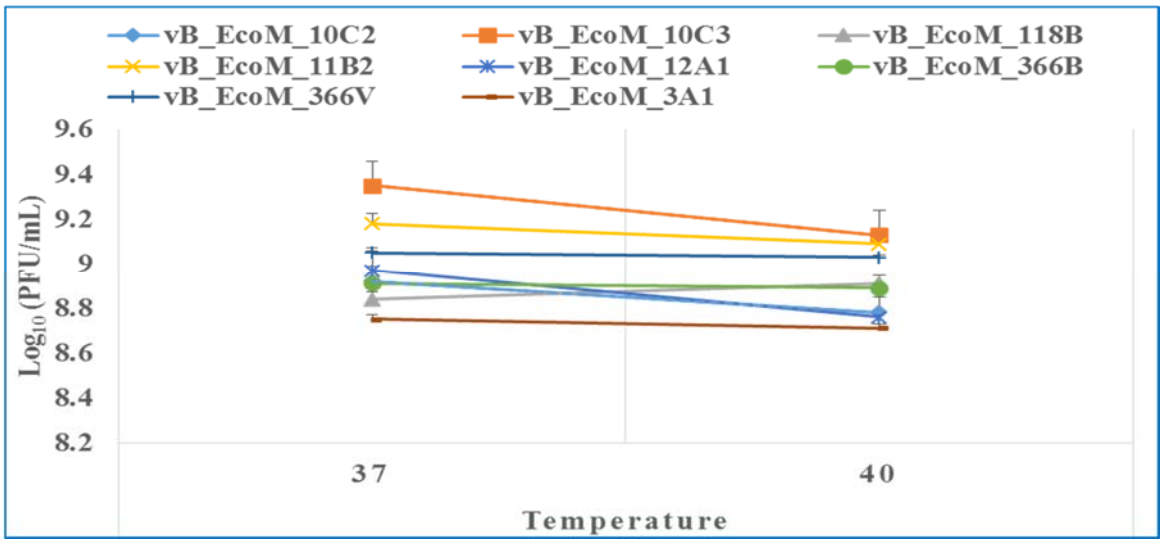


Figure 5.7: Survival and stability of individual phages when exposed to different temperatures for 30 minutes. The error bars represent the standard deviation.

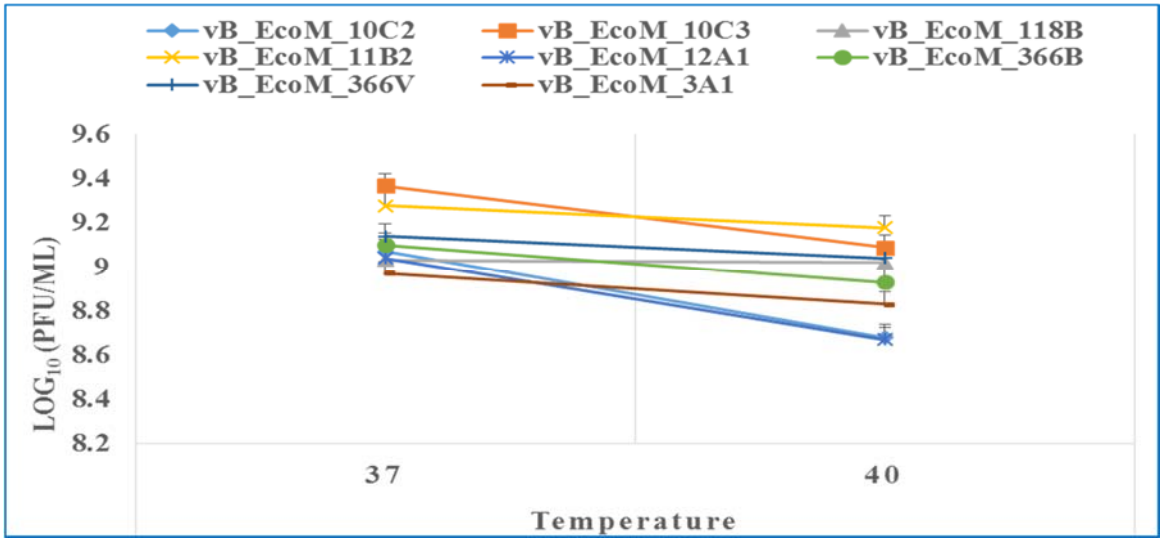
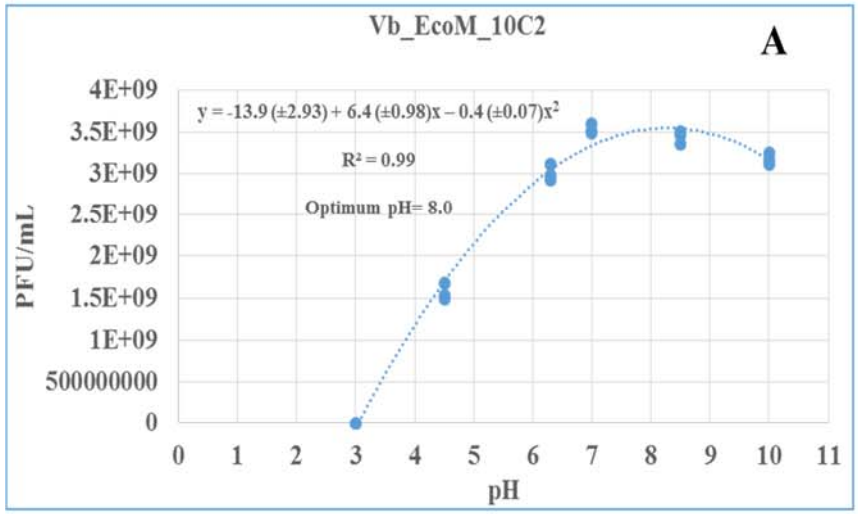


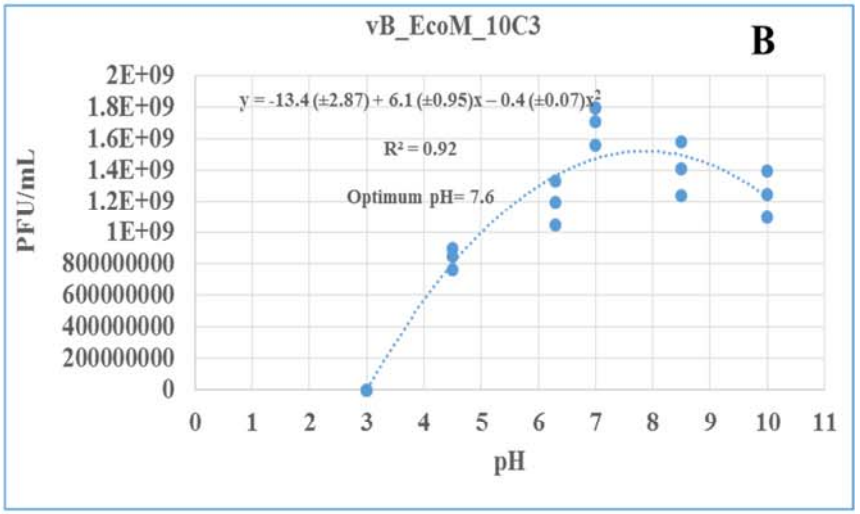
Figure 5.8: Survival and stability of individual phages when exposed to different temperatures for 60 minutes. The error bars represent the standard deviation.

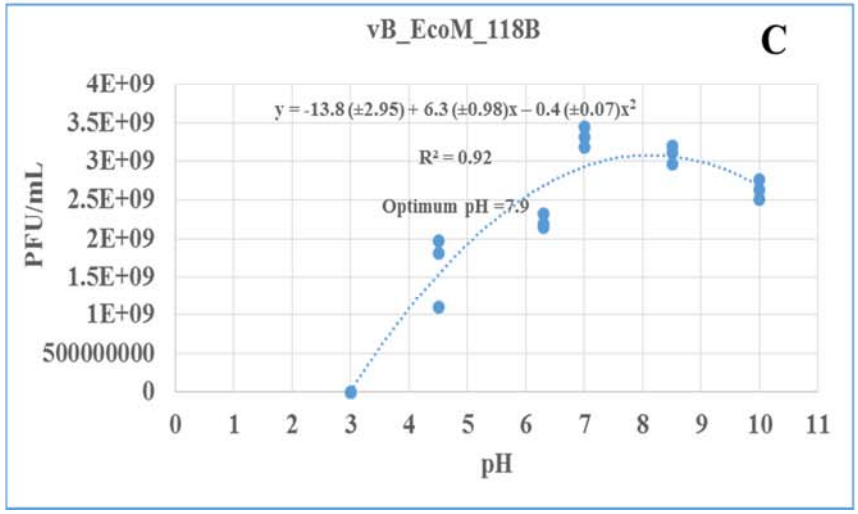
5.3.5. Phage stability and viability against different pH levels

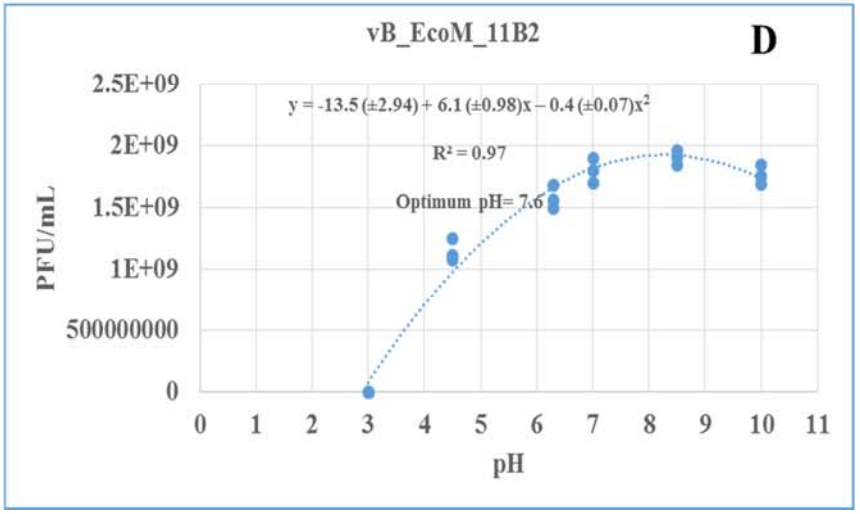
Response surface regression analysis revealed quadratic effects ($p < 0.0001$) of pH on phage stability when incubated for 24 and 48 hours, Figure 5.9 A-H and Figure 5.10 A1-H1, respectively. The pH optima for all the phages ranged from 7.6 to 8.0 with the R^2 values ranging from 0.90 to 1.0 when incubated for 24 hours, Figure 9A-H. Three phages showed maximum

stability at pH 8.0 determined from the following quadratic equations: y = -13.9 (±2.93) + 6.4 (±0.98) x – 0.4 (±0.07) x^2 (vB_EcoM_10C2), y = -13.9 (±3.04) + 6.4 (±1.01) x – 0.4 (±0.08) x^2 (vB_EcoM_366B) and y = -13.9 (±2.97) + 6.4 (±0.99) x – 0.4 (±0.08) x^2 (vB_EcoM_366V). Phages vB_EcoM_10C3 and vB_EcoM_11B2 exhibited maximum stability at pH 7.6, which was determined from the following quadratic equations: y = -13.4 (±2.87) + 6.1 (±0.95) x – 0.4 (±0.07) x^2 and y = -13.5 (±2.94) + 6.1 (±0.98) x – 0.4 (±0.07) x^2, respectively. When incubated for 48 hours, pH optima for phage stability ranged from 7.9 to 8.0 with R^2 values ranging from 0.90 to 1.0, Figure 10A1-H1. Seven phages exhibited maximum stability at the higher (8.0) optimum pH while only one phage showed maximum stability at the lower (7.9) pH determined from the quadratic equation, y = -13.8 (±3.10) + 6.3 (±1.03) x – 0.4 (±0.08) x^2.


Vb_EcoM_10C2
A
y = -13.9 (±2.93) + 6.4 (±0.98)x – 0.4 (±0.07)x²
R² = 0.99
Optimum pH= 8.0
PFU/mL
pH


vB_EcoM_10C3
B
y = -13.4 (±2.87) + 6.1 (±0.95)x – 0.4 (±0.07)x²
R² = 0.92
Optimum pH= 7.6
PFU/mL
pH


vB_EcoM_118B
C
y = -13.8 (±2.95) + 6.3 (±0.98)x – 0.4 (±0.07)x²
R² = 0.92
Optimum pH =7.9
PFU/mL
pH


vB_EcoM_11B2
D
y = -13.5 (±2.94) + 6.1 (±0.98)x – 0.4 (±0.07)x²
R² = 0.97
Optimum pH= 7.6
PFU/mL
pH

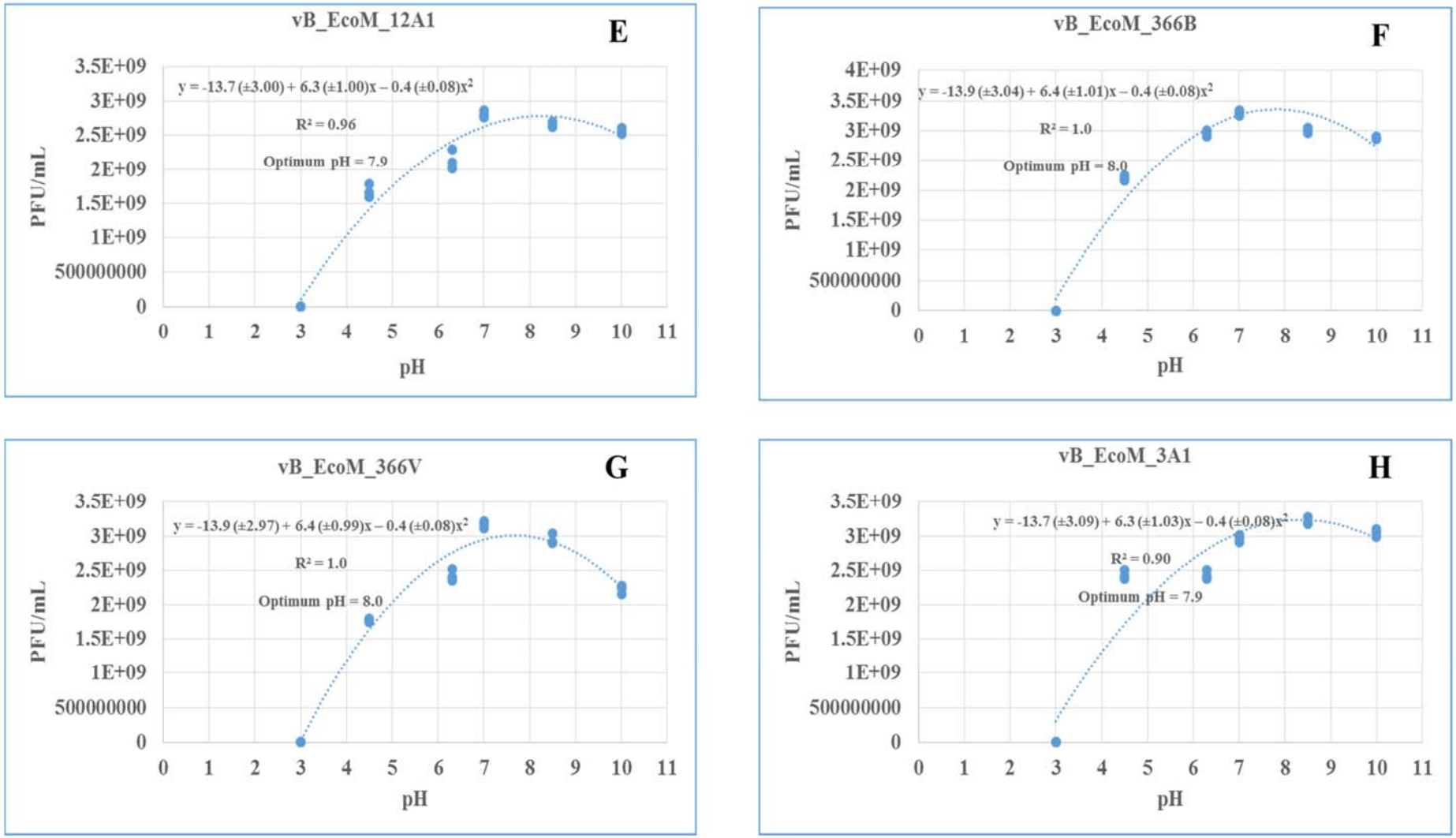
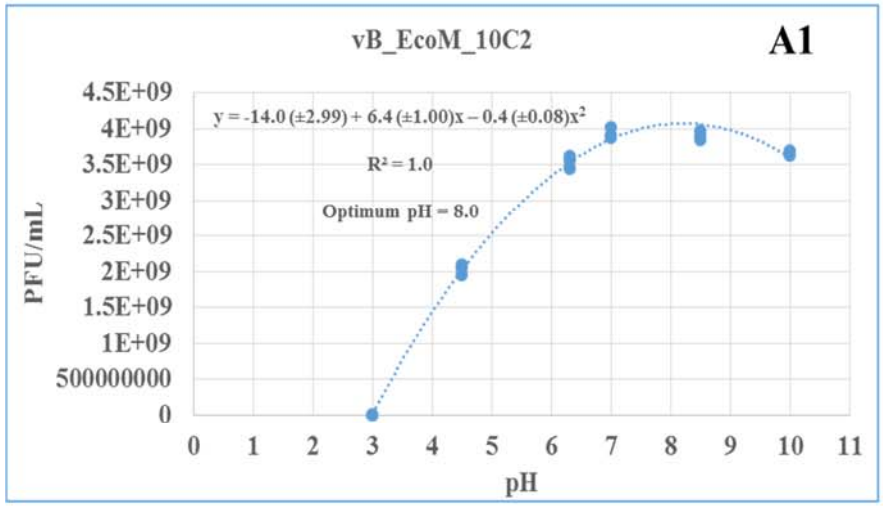
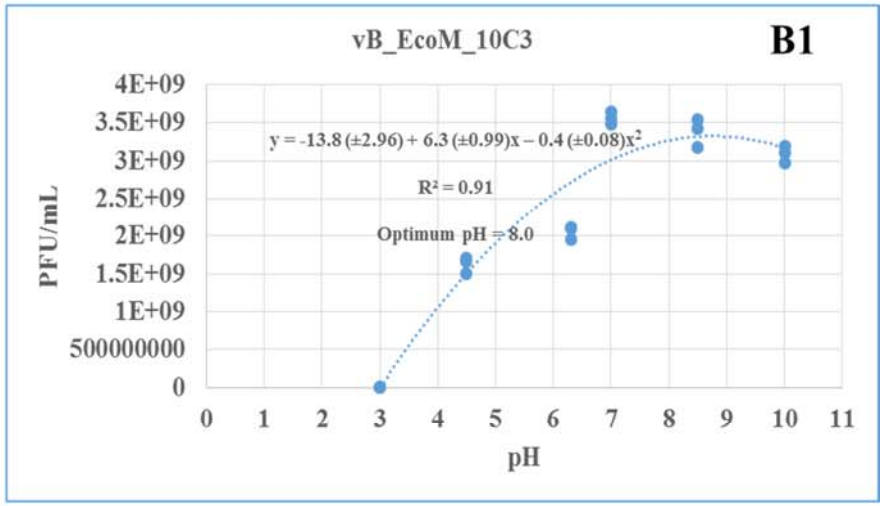
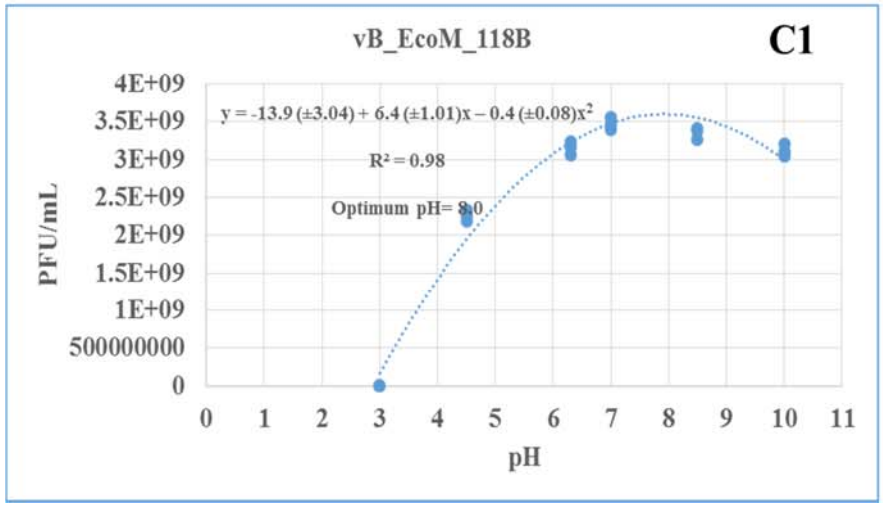
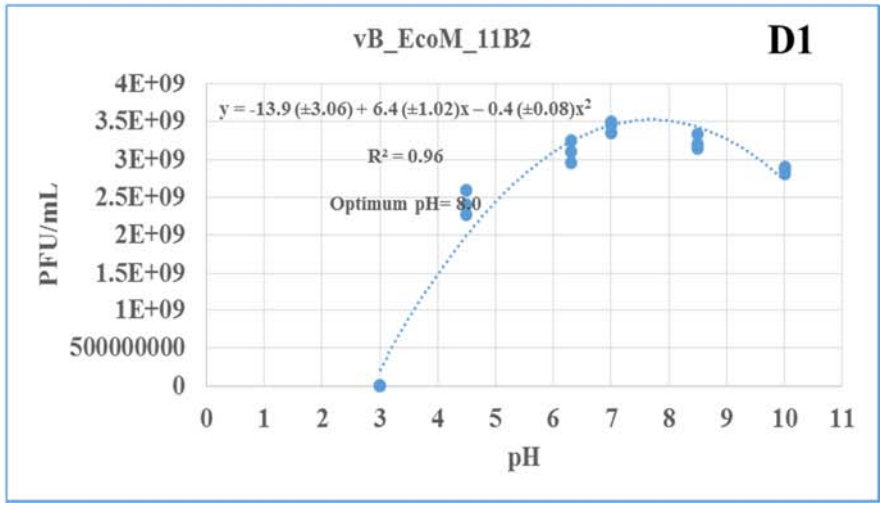


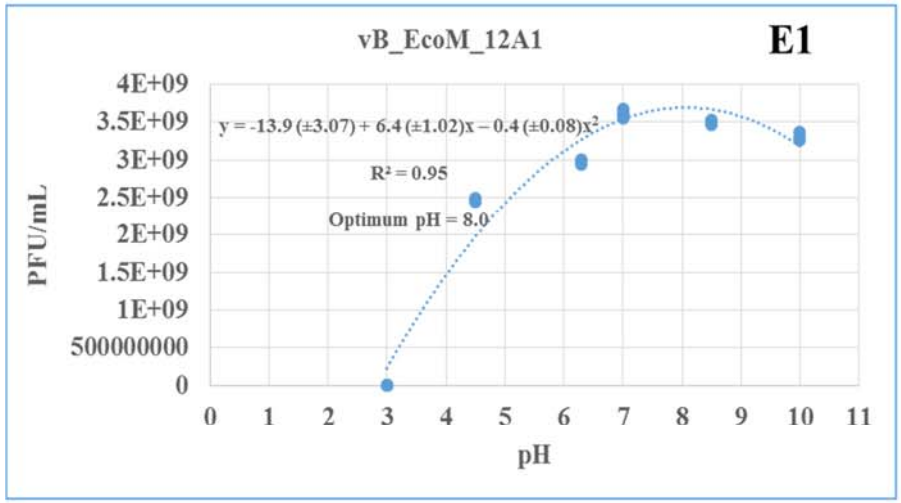
Figure 5.9 (A-H): Relationship between pH (*x*) and stability of phages (Log_{10} PFU, *y*) when incubated at 37 °C for 24 hours.

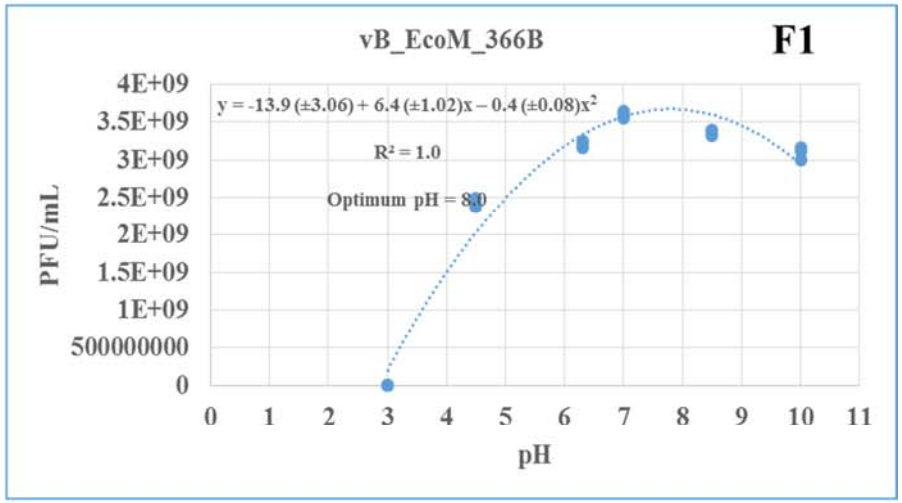

vB_EcoM_10C2
A1
y = -14.0 (±2.99) + 6.4 (±1.00)x – 0.4 (±0.08)x²
R² = 1.0
Optimum pH = 8.0
PFU/mL
pH

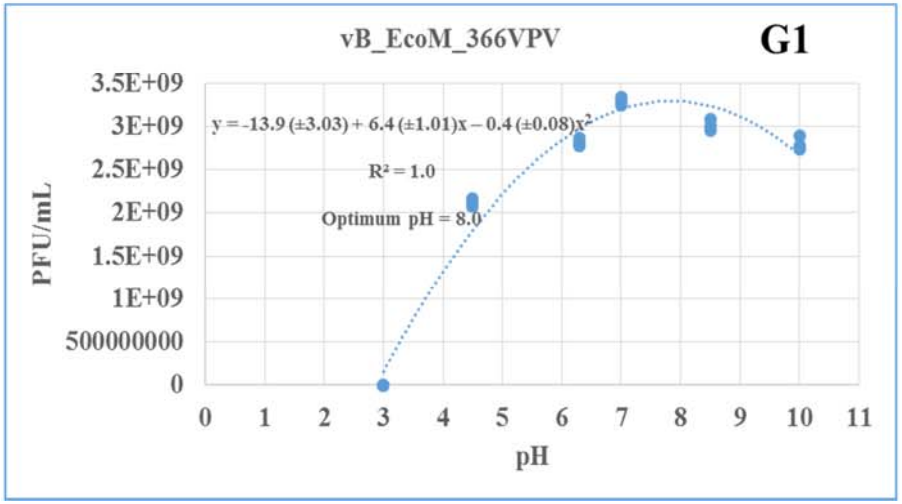

vB_EcoM_10C3
B1
y = -13.8 (±2.96) + 6.3 (±0.99)x – 0.4 (±0.08)x²
R² = 0.91
Optimum pH = 8.0
PFU/mL
pH


vB_EcoM_118B
C1
y = -13.9 (±3.04) + 6.4 (±1.01)x – 0.4 (±0.08)x²
R² = 0.98
Optimum pH= 8.0
PFU/mL
pH


vB_EcoM_11B2
D1
y = -13.9 (±3.06) + 6.4 (±1.02)x – 0.4 (±0.08)x²
R² = 0.96
Optimum pH= 8.0
PFU/mL
pH







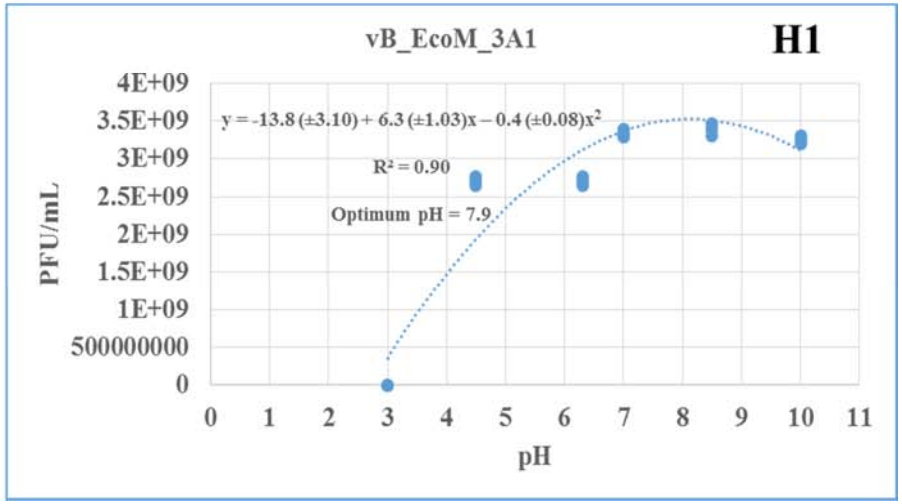
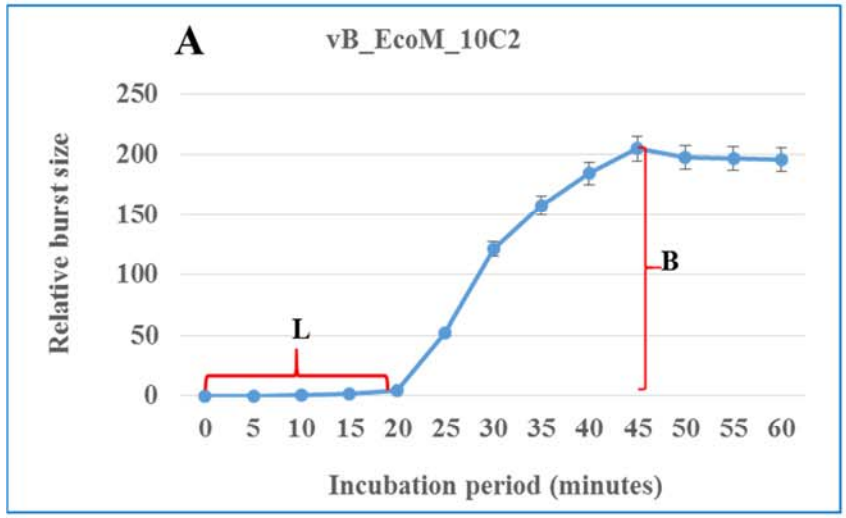
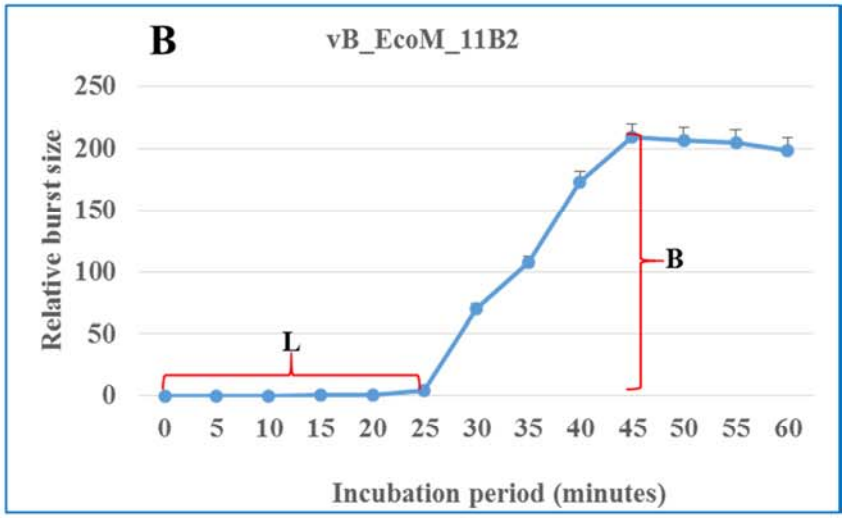


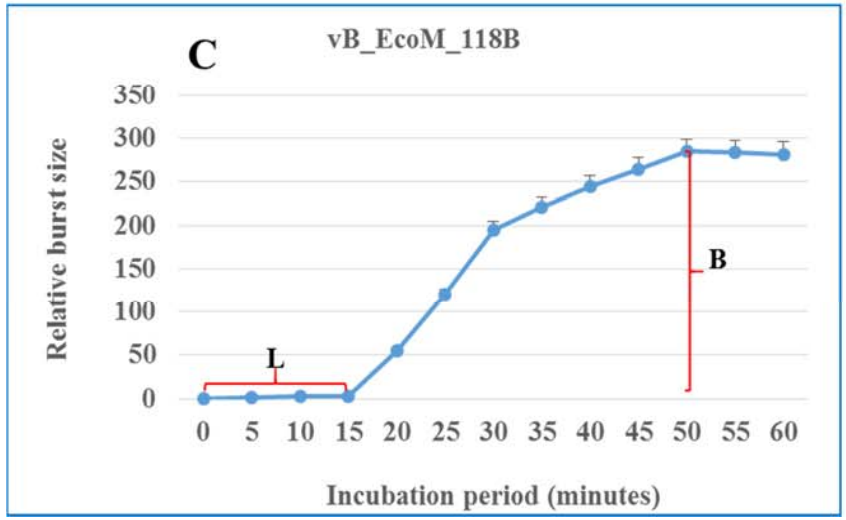
Figure 5.10 (A1-H1): Relationship between pH (*x*) and stability of phages (Log_{10} PFU, *y*) when incubated at 37 °C for 48 hours.

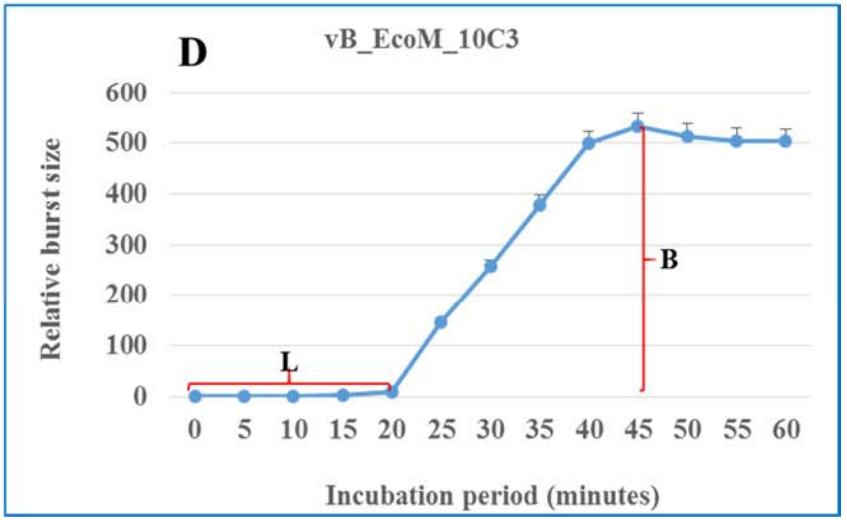
5.3.6. One-step growth curve bacteriophages

One-step growth curve for the eight phage isolates was performed to determine latent period and relative bust size per infected bacteria cell. Figure 5.11 (A-H) depicts phage triphasic growth curve. The latent period for all the phages ranged from 15 to 25 minutes (average = 20 ± 3.8 minutes). Phages vB_EcoM_11B2 and vB_EcoM_3A1 had the longest latent period (25 minutes) while phages vB_EcoM_118B and vB_EcoM_366V had the shortest (15 minutes) latent period. The latent period for the other four phages was 20 minutes. In terms of burst size, phages vB_EcoM_10C3 and vB_EcoM_12A1 had the largest burst size per infected cell (522 PFUs and 367 PFUs, respectively) while phage vB_EcoM_366V had the smallest burst size (91 PFUs) per infected cell.


A
vB_EcoM_10C2
Relative burst size
250
200
150
100
50
0
L
B
0 5 10 15 20 25 30 35 40 45 50 55 60
Incubation period (minutes)


B
vB_EcoM_11B2
Relative burst size
250
200
150
100
50
0
L
B
0 5 10 15 20 25 30 35 40 45 50 55 60
Incubation period (minutes)


C
vB_EcoM_118B
Relative burst size
350
300
250
200
150
100
50
0
L
B
0 5 10 15 20 25 30 35 40 45 50 55 60
Incubation period (minutes)


D
vB_EcoM_10C3
Relative burst size
600
500
400
300
200
100
0
L
B
0 5 10 15 20 25 30 35 40 45 50 55 60
Incubation period (minutes)

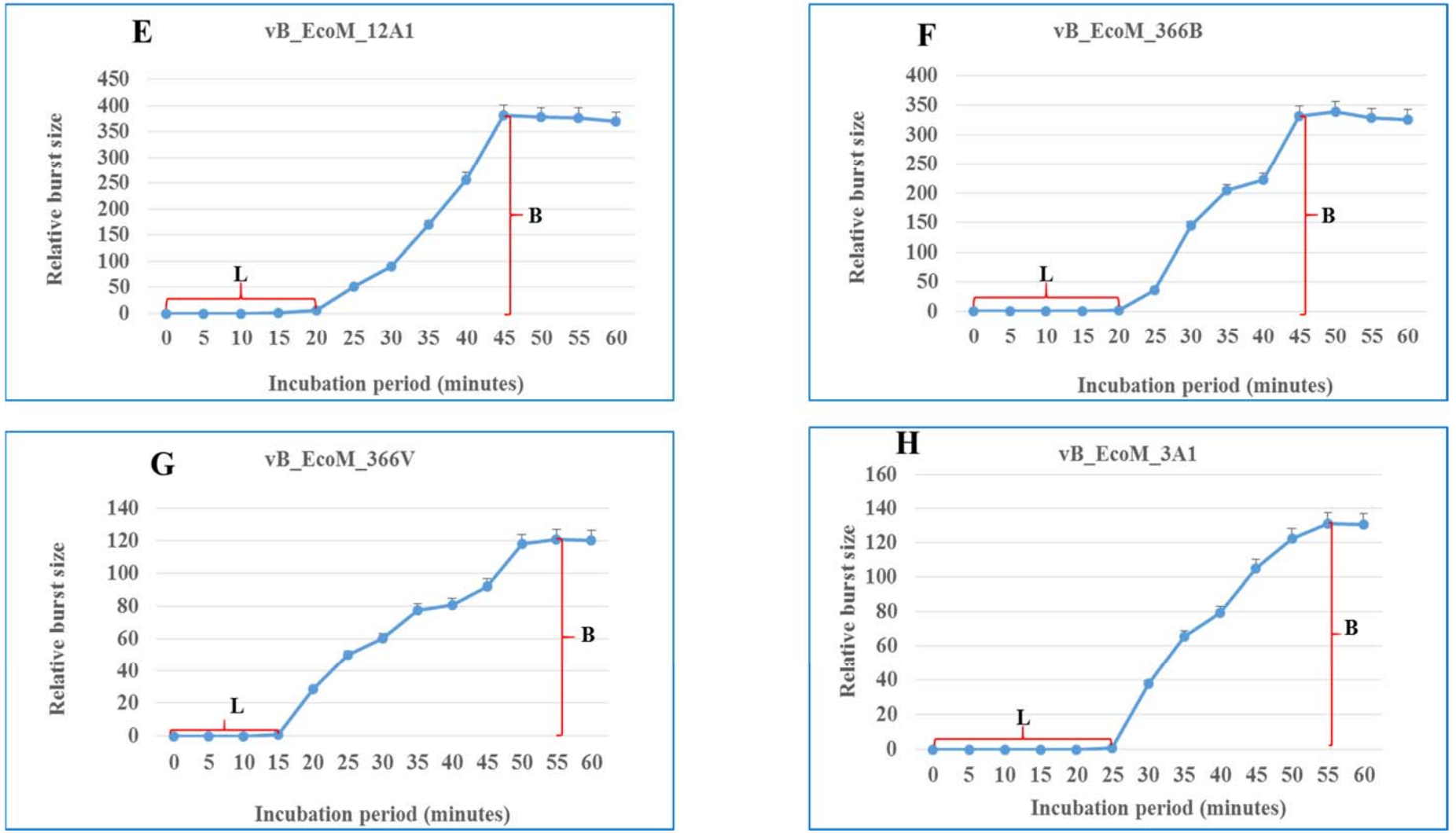


Figure 5.11(A-H): One-step growth curves for eight *E. coli* O177-specific phage isolates. Latent period (**L**), Burst size (**B**). The error bars indicate standard deviation.

5.4. Discussion

The emergence of antibiotic resistance in foodborne pathogens has renewed interest in the possible exploitation of lytic bacteriophages as an alternative biocontrol strategy. The isolation, identification and full characterisation of the bacterial host is a prerequisite for successful isolation of suitable lytic bacteriophages intended for biocontrol purpose (Rao and Lalitha, 2015). Furthermore, reliable, reproducible and efficient methods need to be employed for selection of suitable phage candidates for biocontrol application (Pereira *et al.*, 2016a). Phages intended for biocontrol application should be strongly lytic and have a broad host range (Kazi and Annapure, 2016). Although many studies have isolated and characterised lytic phages infecting *E. coli* O157, *Campylobacter*, *Listeria*, *Pseudomonas*, *Salmonella* and *Vibrio* species (O'flynn *et al.*, 2004; Niu *et al.*, 2014; Perera *et al.*, 2015; Wang *et al.*, 2015; Mahmoud *et al.*, 2017; Zhang *et al.*, 2018; Tang *et al.*, 2019) , no study has isolated and characterised *E. coli* O177-specific phages. Hence, the main objective of this study was to isolate and characterise lytic phages infecting *E. coli* O177 strain.

Thirty-one lytic *E. coli* O177-specific bacteriophage isolates were successfully isolated from cattle faeces using enrichment method. This method was highly efficient and reliable for isolating phages from samples with low concentrations of phages (Akhtar *et al.*, 2014). The phages exhibited clear and discrete plaques with different sizes. The plaque size ranged from small to large (1 mm to2 mm, respectively) size while phage titers ranged from 6.2×10^5 to 3.1×10^{13} PFU/mL. Interestingly, a large proportion (71%) of phage isolates produced large and clear plaques on their preferred hosts. These characteristics were similar to those reported for *E. coli* O157- *Listeria*-, *Pseudomonas*-, *Salmonella*- and *Vibrio*-specific phages (O'flynn *et al.*, 2004; Niu *et al.*, 2014; Perera *et al.*, 2015; Mahmoud *et al.*, 2017; Zhang *et al.*, 2018). Furthermore, phage titers ranged from 6.2 × 105 to 3.1×10^{13} PFU/mL. In addition, it was

noted that the more the phages were purified, the more they produced clear plaques on their respective host bacteria. From a biocontrol point of view, phages with high titer are considered as ideal candidates for biocontrol application (Snyder *et al.*, 2016; Harada *et al.*, 2018). Eight phages were selected for further analysis. The selection criteria were based on the lytic profiles, plaque clarity and size.

Lysis capacity is a desirable biological characteristic of a phage, which is defined by what bacterial genera, species and strains it can infect (Kutter, 2009). For therapeutic purpose, lytic phages should have a relatively wide host range and be able to infect multiple species within a given genus (Pereira *et al.*, 2016b; Yang *et al.*, 2019) . Therefore, determining phage host range is an important criteria for selecting lytic phages for biocontrol purposes (El-Dougdoug *et al.*, 2019). In this study, spot test was used to determine phage host range. Phages were capable of *E. coli* strains from two different categories (environmental atypical enteropathogenic *E. coli* O177 and shiga toxin producing *E. coli* (*E. coli* O26 and *E. coli* O157). Clear plaques were predominantly observed on *E. coli* O177 and *E. coli* O26 serotypes. Interestingly, three phages exhibited clear plaques on *E. coli* O177, *E. coli* O26 *E. coli* O157 strains, suggesting that these phages were polyvalent. Despite this, no phage could infect all the ATCC strains and environmental *Salmonella* species. This could be attributed to the fact that ATCC strains and *Salmonella* species may be devoid of specific receptors for phage attachment. Moreover, host-specificity is regarded as a desirable characteristic for potent phages application, particularly in live animals to ensure that they have little or no impact on the beneficial gut microflora (Tomat *et al.*, 2014; Patel *et al.*, 2015; Akhtar *et al.*, 2017; Huang *et al.*, 2018).

Although spot test is widely used to determine phage host range, several factors such as specific host strain may affect efficiency of phages and thus it is important to evaluate relative

efficiency of plating on various susceptible hosts (Kutter, 2009). In this study EOP assay was performed to assess the lytic efficiency in three different *E. coli* serotypes. Based on EOP analysis, phages revealed high efficiency (EOP ≥ 0.5) on *E. coli* O177 strain. Although spot test results showed that all the phages produced clear plaques on *E. coli* O177 and O26 strains, all the phages exhibited medium to low efficiency of plating (EOP < 0.5) on *E. coli* O26 serotype. This implied that phages isolated in this study were highly specific to *E. coli* O177 strain.

Negative staining procedure is commonly used for transmission electron microscopy analysis. This test is relatively simple and reliable and thus allows for quick observation of physical, structural dimension, identification and classification of phages (Williams and Carter, 1996; Goldsmith and Miller, 2009). Based on TEM analysis results, all the eight phage isolates revealed similar morphotype. Structurally, the phages had icosahedral head, neck attached to a long contractile tail with tail fibres connected to the baseplate. The icosahedral head of the phages ranged from 81.2 ± 6 nm to 95.6 ± 3 nm in size while the contractile tails ranged from 118.1 ± 0.3 nm to 135 ± 2 nm. Based on these characteristics, phage isolates were classified under the order Caudovirales and *Myoviridae* family (Ackermann, 2009). Moreover, these characteristics were similar to those of T1-7-like *E. coli* phage (Truncaite *et al.*, 2012; Wang *et al.*, 2015; Harada *et al.*, 2018). In addition, most of phages reported in the previous studies belonged to *Myoviridae, Padoviridae* and *Siphoviridae* families (Rao and Lalitha, 2015; Stalin and Srinivasan, 2017; Harada *et al.*, 2018). Given that *Myoviridae* family contains double-stranded DNA phages, all the eight phages were further classified under linear double-stranded DNA phages (Harada *et al.*, 2018). The tail fibres contain proteins, which help the phage to recognise their specific receptors on the bacteria cell wall and thus restrict the phage from

binding to non-specific bacteria cell (Singh *et al.*, 2010). This explains the host specificity of the phages isolated in this study.

External factors such as pH and temperature may influence the stability and infectivity of the phages (Yin *et al.*, 2019). These factors may fluctuate, particularly in live animals because of diet and/or ambient temperature. In view of this, phages intended for biocontrol application, particularly in live ruminants, must be tested against an appropriate range of pH and temperature. For this reason, the effect of exposure to different temperatures (37 °C and 40 °C) for different times on infectivity and stability of eight phages was evaluated. Given that complete bacterial lysis by phage takes 20 to 40 minutes (Perera *et al.*, 2015), phage growth at different temperatures was monitored after 10, 30 and 60 minutes. Furthermore, the incubation temperatures were selected because the temperature in the digestive system of the ruminant ranges from 37 to 40 °C. The ability of phages to survive at these temperatures suggests that they can be applied in live animal as biocontrol agents. When incubated at 37 °C, phages revealed significant growth rate at each time point. Phages vB_EcoM_10C3, vB_EcoM_11B2 and vB_EcoM_366V revealed fastest growth rate while phage vB_EcoM_3A1 showed the slowest growth rate from 10 to 60 minutes. These results are similar to those reported in the previous studies (El-Dougdoug *et al.*, 2019).

Phages revealed variable growth patterns when exposed to 40 °C. Generally, phages showed decline in growth rate at 40 °C when compared to their growth rate at 37 °C. When exposed to 40 °C for 10 to 30 minutes, seven phages exhibited significant growth rate. However, one phage revealed decline in growth rate after 30 minutes while two vB_EcoM_10C2 and vB_EcoM_10C3 only exhibited a decline in growth from 30 to 60 minutes at 40 °C. This demonstrates that these three phages were less stable at high temperature. Therefore, their

application in live animals is limited because the rumen temperature is 39 °C. Despite this, the other five phages were fairs stable at 40 °C, suggesting they may be suitable for biocontrol application in live animal.

The response surface regression analysis revealed significant relationship between pH and phage stability. The optimal pH for phages at different incubation times ranged from pH 7.6 to 8.0 (24 hours) and pH 7.9 to 8.0 (48 hours). When incubated for 24 and 48 hours, all phages exhibited similar growth trends and survival over a wide range of pH 4.5-10.0. Despite this, all the phages were sensitive to low pH (3.0) with no activity being observed after 24 and 48 hours of incubation at this pH. This is consistent with previous studies, which reported that exposure of phages to pH 3.0 and below significantly reduced the viability and stability of phages (Hu *et al.*, 2016; Stalin and Srinivasan, 2017). The optimum pH for all the phages 7.6 to 8.0, phages revealed good stability even at lower pHs of 6.3 and 7.0, which encompasses rumen pH values (6.5 to 6.9). This indicates their potential to be used in ruminants to reduce *E. coli* O177 before slaughter.

The stability and infectivity of phages were also evaluated against different pH levels. When incubated for 24 and 48 hours, all phages exhibited similar growth trends and survival over a wide range of pH. However, phages were sensitive to low pH (3.0) and no activity was observed after 24 and 48 hours of incubation at this pH. This is consistent with the previous studies, which reported that exposure of phages to pH 3.0 and below significantly affect the viability and stability of phages (Hu *et al.*, 2016; Stalin and Srinivasan, 2017). Although phages were active and stable at different pH levels (4.5 to 10.0), maximum growth was observed at pH 7.6 to 8.0. The quadratic polynomial regression analysis revealed significant relationship between pH and phage stability. The optimal pH for phages at different times ranged from pH

7.6 to 8.0 (24 hours) and pH 7.9 to 8.0 (48 hours). Although the optimum pH for all the phages was high, phages were stable at pH 6.3 and 7.0, which fall within the rumen pH range (6.5 to 6.9). Considering that rumen houses different microbes including *E. coli* O177 strain, application of these phages may reduce microbial load, particularly in ruminants.

Phage latent period and burst size are important parameters to be consider when selecting phages for biocontrol purposes (Niu *et al.*, 2009). Phages with short latent period and large burst size are more effective in inactivating bacteria and are thus considered to be suitable for biocontrol application (Duc *et al.*, 2018). One-step growth curve revealed that the eight phages have different patterns of growth, suggesting that they have distinct genotypes. They displayed outstanding characteristics such as short latent period and large bust sizes, which make them attractive for the control of *E. coli* O177 strain. The latent period of phages ranged from 15 to 25 minutes while the burst size ranged from 91 to 522 PFU/host cell. In addition, the average latent period for all the phages was 20 ± 3.8 minutes while the burst size was 260 ± 144 PFU/host cell. These results were consistent with those reported previously (Duc *et al.*, 2018). Two phages, vB_EcoM_118B and vB_EcoM_366V, had the shortest latent period (15 minutes) while vB_EcoM_11B2 and vB_EcoM_3A1 had the longest latent period (25 minutes). Phages vB_EcoM_10C3, vB_EcoM_12A1 andvB_EcoM_366B had the largest burst size per infected cell (522, 367 and 331 PFU/host cell, respectively). Interestingly, these three phages also showed broad host range in the spot test. This demonstrates that these phages are better suitable for biocontrol application (Kalatzis *et al.*, 2016).

5.5. Conclusion

Lytic bacteriophages infecting *E. coli* O177 environmental strain were successfully isolated in this study. Furthermore, phages were capable of infecting three *E. coli* strains from two different categories, atypical enteropathogenic *E. coli* (*E. coli* O177) and shiga toxin producing *E. coli* (*E. coli* O26 and O157). Despite this, no phage could infect ATCC strains and environmental *Salmonella* species tested. Transmission electron microscopic analysis revealed a similar morphotype for all the eight phage isolates and were all classified under *Myoviridae* family. Furthermore, phages were stable over a wide range of pH of 4.5 to 10.0, with the optimum growth ranging between pH 7.6 and pH 8.0 and temperature (37 °C and 40 °C). Based on these characteristics, phages isolated in this study are considered as potential candidates for *in vivo* control of *E. coli* O177 strain. However, further studies using appropriate *in vitro* and *in vivo* models are required to evaluate the efficacy of *E. coli* O177-specific phages in reducing *E. coli* O177 in live animals and meat products.

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CHAPTER SIX

EFFICACY OF NOVEL PHAGES FOR CONTROL OF MULTI-DRUG RESISTANT *ESCHERICHIA COLI* O177 ON ARTIFICIALLY CONTAMINATED BEEF

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CHAPTER SIX

EFFICACY OF NOVEL PHAGES FOR CONTROL OF MULTI-DRUG RESISTANT *ESCHERICHIA COLI* O177 ON ARTIFICIALLY CONTAMINATED BEEF

(This chapter is under review in the journal "*Food Microbiology*", with authors Peter Kotsoana Montso, Victor Mlambo and Collins Njie Ateba)

Abstract

Foodborne infection remains a major cause of morbidity and mortalities worldwide. The current food safety interventions have serious limitations and drawbacks in mitigating foodborne pathogens in food. The aim of this study was to determine the efficacy of individual phages and their cocktails in reducing *E. coli* O177 viable cells on artificially contaminated meat. Furthermore, individual phages and cocktails were assessed for their ability to prevent biofilm formation as well as to degrade established biofilm structures. Phage cocktail exhibited high activity at low titer than individual phages. Both individual phages and their cocktails were capable of reducing *E. coli* O177 cell count on artificially contaminated beef. Phages reduced bacteria cell count over a seven-day incubation period at 4 °C. Two individual phages, vB_EcoM_12A1 and vB_EcoM_3A1 and three cocktails, T3, T4 and T6, reduced bacteria cell count to below detection limit (1.0 $\log_{10}$ CFU/mL). On average, individual phage resulted in *E. coli* O177 cell count reduction of 7.2 $\log_{10}$ CFU/mL while phage cocktails caused a reduction of 3.9 $\log_{10}$ CFU/mL on day one. Relative reduction percentage ranged from 73 to 100% and 32 – 100% (individual phages and cocktails, respectively). On day three and seven, bacterial cell count increased on meat treated with individual phages (vB_EcoM_10C2, vB_EcoM_10C3, vB_EcoM_118, vB_EcoM_11B, vB_EcoM_366B, vB_EcoM_366V) and

phage cocktails (T1, T2 and T5). However, bacterial cell counts in treated meat samples were significantly lower than the controls. Individual phages and phage cocktails revealed potential in inhibiting the growth of *E. coli* O177 biofilm formation with the later showing greater potency in destroying pre-formed biofilms than the former. This finding suggests that phage cocktails developed in this study can be used for biocontrol of *E. coli* O177 on meat at storage conditions to improve food safety.

Keywords: *E. coli* O177; phage cocktails; biofilm; bio-control; food safety.

6.1. Introduction

Food contamination may occur at different levels along the food chain; farms, markets and/or households (Sillankorva *et al.*, 2012). *Escherichia coli*, *Campylobacter*, *Listeria* and *Salmonella* species are common foodborne pathogens implicated in foodborne outbreaks (Sillankorva *et al.*, 2012). More than 600 million people become ill and 420 000 die each year due to foodborne infections (WHO, 2017). Besides illnesses, hospitalisation and deaths, foodborne pathogens may obtrude financial burden on local and international trade, resulting in economic losses, especially for the exporting countries (Sillankorva *et al.*, 2012; Endersen *et al.*, 2014). For instance, the cost estimates associated with a listeriosis outbreak in South Africa in 2017-2018 exceeded US$ 260 million (Olanya *et al.*, 2019). Monthly production losses and hospitalisation costs were estimated at US$10.4 and US$ 15 million, respectively (Olanya *et al.*, 2019).

Various chemical, physical and biological methods have been developed and employed to promote food safety (Duc *et al.*, 2018). Despite this, food safety continues to be a challenge due to changes in production system, international trade and consumers' life style (Sillankorva

et al., 2012; Tan *et al.*, 2014). Furthermore, the current food safety interventions have many drawbacks such as corrosive effects on food processing plants and changes in organoleptic properties of food (Sillankorva *et al.*, 2012; Endersen *et al.*, 2014). Residues from chemical interventions may also have detrimental effects on human health as well as the environment. In addition, these chemical agents may precipitate biofilm formation and antimicrobial resistance. It is against this background that efforts have been made to search for novel, safe and sustainable methods to ensure food safety. Bacteriophage therapy is a promising natural and green technology for food preservation and safety (Sillankorva *et al.*, 2012; Endersen *et al.*, 2017; Harada *et al.*, 2018). Lytic bacteriophages are regarded as potential candidates to combat microbial contamination in the food industry. The use of lytic phages for the treatment of foodborne pathogens, particularly in live animals and food commodities, have the potential to replace conventional methods (O'flynn *et al.*, 2004; Rozema *et al.*, 2009; Raya *et al.*, 2011; Tomat *et al.*, 2014; Endersen *et al.*, 2017; Duc *et al.*, 2018). In addition, lytic phages kill multi-drug resistant (superbugs) pathogens and degrade their biofilm structures. Phages are unlikely to induce negative effects in food, environment, humans and animals (Duc *et al.*, 2018). Currently, a number phages such as ListShield™, EcoShield™, SalmoFresh™ and Salmonelex™ are considered safe to be used in food industry to ensure food safety (Duc *et al.*, 2018; Harada *et al.*, 2018).

Despite the fact that phage-based biocontrol approach is considered as an alternative for prevention and control of antimicrobial resistant foodborne pathogens, development of phage resistance in single phage therapy is a cause for concern (Labrie *et al.*, 2010; Patel *et al.*, 2015). Phage resistance may occur as a result of alteration and/or loss of bacterial cell surface receptors required for phage attachment, gene mutation and production of modified restriction endonucleases, which degrade the phage DNA (Chan *et al.*, 2013; Tsonos *et al.*, 2014). The

limitations of single phage application may be overcome by the use of phage cocktails (Chen *et al.*, 2018). Phage cocktails target different host cell receptors and can be used to circumvent the development of phage-resistant bacterial mutants and thus broaden the host range (Chan *et al.*, 2013; Bai *et al.*, 2019). Several studies demonstrated that phage cocktails have potential in reducing phage resistant mutants (Perera *et al*., 2015; Chen *et al.*, 2018; Bai *et al.*, 2019).

Despite the potential and usefulness of bacteriophages, application of phages to improve food safety is still in its infancy (Doyle and Erickson, 2012). In addition, the extent to which *E. coli* O177-specific bacteriophages can reduce *E. coli* O177 in food products has not been elucidated. Therefore, this study was designed to evaluate the efficacy of individual phages and phage cocktails in reducing cell counts, preventing biofilm formation and destroying existing biofilm structures of *E. coli* O177 on artificially contaminated beef.

6.2. Materials and methods

6.2.1. Bacterial culture and phage

Escherichia coli O177 strain used for *in vitro* experiment was isolated from cattle faeces and was identified and confirmed through amplification of 16S rRNA, *rmlB* and *wzy* gene sequence analysis (GenBank Accession numbers of representative samples, MH389799, MH389800 and MH389801) (Chapter 3). The isolates were preserved in 20% (v/v) glycerol and stored at −80 °C. Before use, the isolates were resuscitated on sorbitol MacConkey agar supplemented with 1 mg/L potassium tellurite and aerobically incubated at 37 °C for 24 hours. Fresh colonies were transferred into 15 mL sterile falcon tube containing 5 mL nutrient broth. The samples were incubated at 37 °C for 24 hours. After incubation, aliquot of 100 µL of overnight culture was transferred into 10 mL Tryptic soya broth (TSB) and incubated in a shaking incubator at 37 °C

for 3 hours until optical density of (OD_{630mn} = 0.5) corresponding to 1 x 10^8 CFU/ mL was obtained.

Eight phage isolates belonging to *Myoviridae* family were isolated from cattle faeces samples (Chapter 5). Phages were propagated using *E. coli* O177 as previously described (Sambrook and Russell, 2001). Subsequently, plaque assay was performed to determine the titer of each phage. Phage stock was used to determine individual phages virulence capabilities and to develop phage cocktails.

6.2.2. Optimal multiplicity of infection and lytic capabilities of individual phages using microplate virulence assay

Lytic capabilities of individual phages was assessed against nine (9) multi-drug resistant *E. coli* O177 isolates using microplate virulence assay as previously described (Niu *et al.*, 2009). Briefly, 180 µL of mTSB was dispensed into 96-well polystyrene plates. In the first row (A-1-8 wells), 20 µL of each phage isolate (stock, 1 x 10^8 PFU/mL) was inoculated in duplicates and serially diluted in 10-folds (10^{-1} to 10^{-8}). Subsequently, aliquot of 20 µL of overnight culture of each *E. coli* O177 isolate (1 x 10^8 CFU/mL) was inoculated into each well (row: 1-8; column: A-H). The ninth and tenth wells served as negative control and they were only inoculated with host bacteria (20 µL) without phage treatment. The eleventh and twelfth wells (served as positive/blank control) were inoculated with mTSB (20 µL) with both phage and bacteria. The plates were incubated at 37 °C for 5 hours. After incubation, phage activity was assessed by measuring Optical Density (OD; 630_{nm}) using microplate reader (Model: MB580) (Fridholm and Everitt, 2005). The highest dilution that resulted in complete lysis of the host bacteria was recorded. The data was used to calculate multiplicity of infection (MOI). The MOI for each phage was determined by dividing the initial concentration of phage (PFU/mL) in the inoculum

with the initial concentration of host bacteria (CFU/mL). The sensitivity of the bacteria isolates to phages was defined as susceptible (MOI < 100) or resistant (MOI ≥ 100) as previously described (Niu *et al.*, 2009). Sensitivity to phages was further categorised as extremely susceptible (MOI < 0.01); highly susceptible (0.01 ≤ MOI < 1); moderately susceptible (1 ≤ MOI < 10) and minimally susceptible (10 ≤ MOI < 100) (Niu *et al.*, 2009). The following equation was used to calculate MOI:

$$\text{Equation: MOI (for equal volumes)} = \frac{\text{No of PFU/ mL}}{\text{No of CFU/ mL}}$$

6.2.3. Optimal MOIs and lytic capabilities of phage cocktails using microplate virulence assay

Phage cocktails were produced by combining equal volumes of each individual phage stock solution (1 x 10^8 PFU/mL) and 32 phage cocktails with different combinations were obtained. Before starting the experiment, phage cocktail titers were standardised by serial dilution to ensure equal final concentration of each phage. Microplate virulence assay was performed to evaluate lytic capability of different combinations of phage cocktails against three multi-drug resistant *E. coli* O177 isolates as described in Section 6.2 above (Niu *et al.*, 2009). Briefly, 180 µL of mTSB was dispensed into 96 wells polystyrene plates. In the first row (A-1-8), 20 µL of phage cocktail stock (1 x 10^8 PFU/mL) was inoculated in duplicates and serially diluted in 10-folds by transferring 20 µL each to subsequent wells, producing eight 10-fold serial dilutions (10^{-1} to 10^{-8}) of phages. Subsequently, aliquot of 20 µL of overnight culture of each *E. coli* O177 isolate (10^8 CFU/mL) was inoculated into each well (1-8). The ninth and tenth wells were inoculated with host bacteria (20 µL) without phage (served as negative control). The eleventh and twelfth wells were inoculated with mTSB (20 µL) and no phage or bacteria was added. These wells served as positive control for phage activity. The plates were incubated at

37 °C for 5 hours. After incubation, phage activity was evaluated by measuring OD_{600nm} using microplate reader (Model: MB580). The MOI for each phage cocktail and sensitivity of bacteria to phages were determined as previously described (Niu *et al.*, 2009).

6.2.4. *In vitro* efficacy of individual phages and phage cocktails in reducing *E. coli* O177 on experimentally contaminated beef

6.2.4.1. Sample preparation and experimental design

Two experiments were performed simultaneously to evaluate the efficacy of individual phages and phage cocktails in reducing multi-drug resistant *E. coli* O177 strain on artificially contaminated beef. Eight individual phages and six phage cocktails (selected based on lytic capabilities, host range and the best MOI against multi-drug resistant *E. coli* O177 strain) were used in this experiment. Beefsteak (5.5 kg) was purchased from local supermarket and the meat was placed in a cooler box containing ice packs and transported to the Molecular Microbiology Laboratory, NWU. The meat was processed within an hour of collection. Briefly, the samples were processed in the laminar flow hood and the meat was aseptically cut into small pieces, 3cm x 3cm square (approximately 25 g) using a sterile pair of scissors and rat tooth forceps. For individual phage treatment, a total of 108 meat samples were placed in the sterile petri-dishes (90 mm) and were randomly assigned in triplicates to nine treatments [eight individual phages, (1×10^8 PFU/mL) and one control (lambda diluent)]. For phage cocktails treatment, 72 meat samples were placed in sterile petri-dishes (90 mm) and were randomly assigned in triplicates to seven treatments [six phage cocktails, (1×10^8 PFU/mL) and one negative control (lambda diluent)] and one control (lambda diluent buffer)]. The remaining 1 kg of the meat sample was used to determine the pH as well as screening for the presence of background *E. coli* and/or any microbial contamination.

6.2.4.2. Sample inoculation, bacteriophage application and bacteria enumeration

A 100 µL of exponential phase (*E. coli* O177; OD_{600nm} = 0.5) culture was pipetted onto the surface of each meat sample to simulate natural contamination and the plates were left in the laminar flow hood for 10 minutes to allow the bacteria to attach to the meat. Subsequently, 1000 µL of each phage and phage cocktail (1 x 10^8 PFU/mL, to achieve MOI of 10) was pipetted on the surface of the samples. For negative control, samples were treated per experiment with 1000 µL lambda diluent buffer (pH 7.2) (without phages). The samples were left in the laminar flow hood for 10 minutes to allow the phages to attach to the host bacteria. The samples were then placed in a sterile container and covered with a clean wrap to prevent contamination. The samples were incubated for seven days at 4 °C to simulate the storage temperature. Samples were analysed after 0, 1, 3 and 7 days of incubation to determine the number of viable *E. coli* O177 cell remaining. The purpose of analysing the samples at day 0 was to confirm the presence inoculum on both treated and untreated samples.

6.2.4.3. Enumeration of viable *E. coli* O177 cells

The remaining bacterial cells on experimentally contaminated beef after exposure to phages were analysed using plate count method. At indicated time points, (day 0, 1, 3 and 7) beef samples treated with both individual phages and phage cocktails including their respective negative controls were withdrawn separately and transferred into 250 mL sterile volumetric flasks containing 50 mL of 0.1% (w/v) peptone water (PW). The flasks were placed on a shaker (200 rpm) for two minutes to facilitate the diffusion of the bacteria into the solution. For enumeration of *E. coli* O177 cell counts for both treated and untreated samples, aliquots of 10 mL were transferred into 50 mL sterile falcon tubes and centrifuged at 12 000 × g for five minutes. The supernatant was discarded and the pellets were resuspended in 10 mL of 0.1% PW. Ten-fold serial dilutions were prepared and aliquots of 250 µL were plated in triplicate on

MacConkey agar plates and the plates were incubated at 37 °C for 24 hours for viable bacterial cell count. After incubation, typical *E. coli* O177 colonies were counted and the results were reported as log CFU/mL.

6.2.5.1. Biofilm formation

Biofilm formation by *E. coli* O177 strain was evaluated using 96-well polystyrene plates as previously described (Stepanović *et al.*, 2000). Eighty-one *E. coli* O177 isolates (selected based on virulence and antimicrobial resistance profiles) and one *Pseudomonas aeruginosa* (ATCC 27853) strain were cultured on MacConkey agar and nutrient agar plates, respectively, and incubated at 37 °C for 24 hours. After incubation, a single colony of each isolate was transferred into 5 mL of sterile nutrient broth in 15 mL falcon tubes. The tubes were incubated at 37 °C for 24 hours. After incubation, 1:100 serial dilutions were prepared and 200 µL was dispensed in triplicates into the 96 well polystyrene plates. The first set of three wells were inoculated with 200 µL of nutrient broth (negative control) and the second set of three wells were inoculated with 200 µL of *Pseudomonas aeruginosa* (ATCC 27853) suspension (positive control; strong biofilm producer). The plates were incubated at 37 °C for 24 and 48 hours. After incubation at each time point, the plates were washed three times with 300 µL of sterile phosphate buffer-saline [PBS: 8 g/L NaCl, 0.2 g/L KCL, 0.2 g/L K_2HPO_4, 2.85 g/L $NaHPO_4$ ・12H2O], (pH 7.4) to remove planktonic cells. After washing, the wells were stained with 200 µL of 0.1% crystal violet solution for 1 hour. Subsequently, the plates were washed five times with 300 µL of sterile PBS. The plates were placed in the laminar flow hood to dry. After trying, 200 µL of 95% ethanol was added to fix the biofilm formation. To check the presence of biofilm formation, the OD_{600nm} of each plate was measured using spectrophotometer. The cut-off value (ODc) was defined as three standard deviation (SD) above the mean OD of the negative control. Biofilm formation was classified into four categories based on the ODs

obtained: OD ≤ ODc, non-adherent; ODc < OD ≤ 2 x ODc, weak biofilm formation; 2 x ODc < OD ≤ 4 x ODc, moderate biofilm formation; and 4 x ODc < OD, strong biofilm formation (Stepanović *et al.*, 2000).

6.2.5.2. Efficacy of lytic individual phages and phage cocktails in preventing biofilm formation by *E. coli* O177

The ability of individual and phage cocktails to prevent biofilm formation by *E. coli* O177 strain was evaluated using the microplate virulence assays in two experiments that were performed separately. Ten *E. coli* O177 isolates (strong biofilm producers) were selected for this experiment as previously described (Stepanović *et al.*, 2000; Endersen *et al.*, 2017), with minor modifications. Briefly, 190 µL of sterile mTSB was dispensed into a 96-well polystyrene plates and as negative control, 10 µL of sterile mTSB was added to first set of three wells. For positive control, 10 µL of *Pseudomonas aeruginosa* ATCC 27853 strain (strong biofilm producer) and *E. coli* O177 isolate (strong biofilm producer from this study) were added separately to the second and third set of three wells, respectively. Instead of phage, 50 µL of sterile lambda diluent solution was added to both negative and positive control wells. A 10 µL of overnight culture of each isolate was added in triplicates to the rest of the wells. For each well (except for both controls), 50 µL of each individual phage stock (1 x 10^8 PFU/mL) and phage cocktail stock (1 x 10^8 PFU/mL) were added separately in triplicate to each host bacteria. The plates were incubated at 25 °C for 24 hours. After incubation, wells were washed three times with 300 µL PBS to remove planktonic cells and subsequently, the wells were stained with 200 µL of 0.1% crystal violet solution for 1 hour. After 1 hour, the stain was removed and the wells were washed five times with PBS. The plates were dried in the laminar flow cabinet. Two hundred microliter of 95% absolute alcohol was added to the wells. Then the OD_{600nm} was

measured using spectrophotometer. The results were interpreted and biofilm formation was classified as described above (Stepanović *et al.*, 2000).

6.2.5.3. Phage treatment to destruct formed-biofilms

The ability of individual phages and phage cocktails to destruct established biofilm structure was assessed using the procedure described previously (Stepanović *et al.*, 2000; Endersen *et al.*, 2017), with some modifications. Ten *E. coli* O177 isolates, strong biofilm producers were selected and overnight cultures were prepared. Briefly, 190 µL of sterile mTSB was dispensed into a 96 well polystyrene plates. A 10 µL of mTSB (negative control) added to the first set of three wells and for positive control, 10 µL of *Pseudomonas aeruginosa* ATCC 27853 strain and *E. coli* O177 isolate (both strong biofilm producer from this study) were added separately to the second and third set of three wells, respectively. A 10 µL overnight culture of each isolate was added in triplicates into the rest of the wells. The plates were incubated at 25 °C for 24 hours to allow the bacteria to form biofilm. After incubation, the media containing planktonic cells was removed and the wells were washed three times with 200 µL of PBS. Subsequently, 150 µL of mTSB was added to each well. Fifty microliter of individual phages and phage cocktails stock (1 x 10^8 PFU/mL) were added separately in triplicate to each host bacteria. Fifty microliter of sterile lambda diluent solution was added to both negative and positive (established biofilm) controls. The plates were incubated at 25 °C for five hours to allow the phages to disintegrate the formed-biofilm in the wells. The medium was removed and the wells were washed three times with 200 µL of PBS. After washing, the plates were stained as described above. After 1 hour, the stain was removed and the wells were washed five times with PBS. The plates were allowed to dry. After drying, 200 µL of 95% absolute alcohol was added to the wells. Then the OD_{600nm} of the mixture was measured using spectrophotometer. The results were interpreted as described above (Stepanović *et al.*, 2000).

6.2.6. Statistical analysis

The data for efficacy of individual phages and phage cocktails in reducing *E. coli* on artificially contaminated beef were converted to log CFU/mL and analysed using the repeated measures of SAS (2010) version 9.3. The PROC GLM procedure of SAS (2010) version 9.3 was used for analysing the data and the least significant difference test was used to separate treatment means. The means were reported at 95% confidence level ($P \leq 0.05$). The GLM model used was:

$$Yij = \mu + D_i + E_{ij}$$

where Y_{ij} is the observation of the dependent variable ij; μ the fixed effect of population means for the variable; D_i the effect of the experimental treatments and E_{ij} the random error associated with observation ij, assumed to be normally and independently distributed.

6.3. Results

6.3.1. Sensitivity of *E. coli* O177 against individual phages

Susceptibility of *E. coli* O177 isolates against lytic *E. coli* O177-specific phages was evaluated using microplate virulence assay. *E. coli* O177 isolates revealed various susceptibility patterns against lytic *E. coli* O177-specific phages, Figure 6.1. Generally, all the isolates were sensitive to phages. *E. coli* O177 susceptibility to phages ranged between 66.7 and 88.9%). Phages were able to inhibit bacterial growth within 5 hours of incubation period. Phage vB_EcoM_10C2 and vB_EcoM_11B2 revealed similar lytic pattern against *E. coli* O177 isolates. The MOI of phages against *E. coli* O177 isolates ranged from 0.00000013 to 7.3, Table 6.1. The average percentage of isolates classified as extremely susceptible (MOI < 0.01) and moderately susceptible ($1 \leq MOI < 10$) to all the phages was 76.4% and 0.2%, respectively.

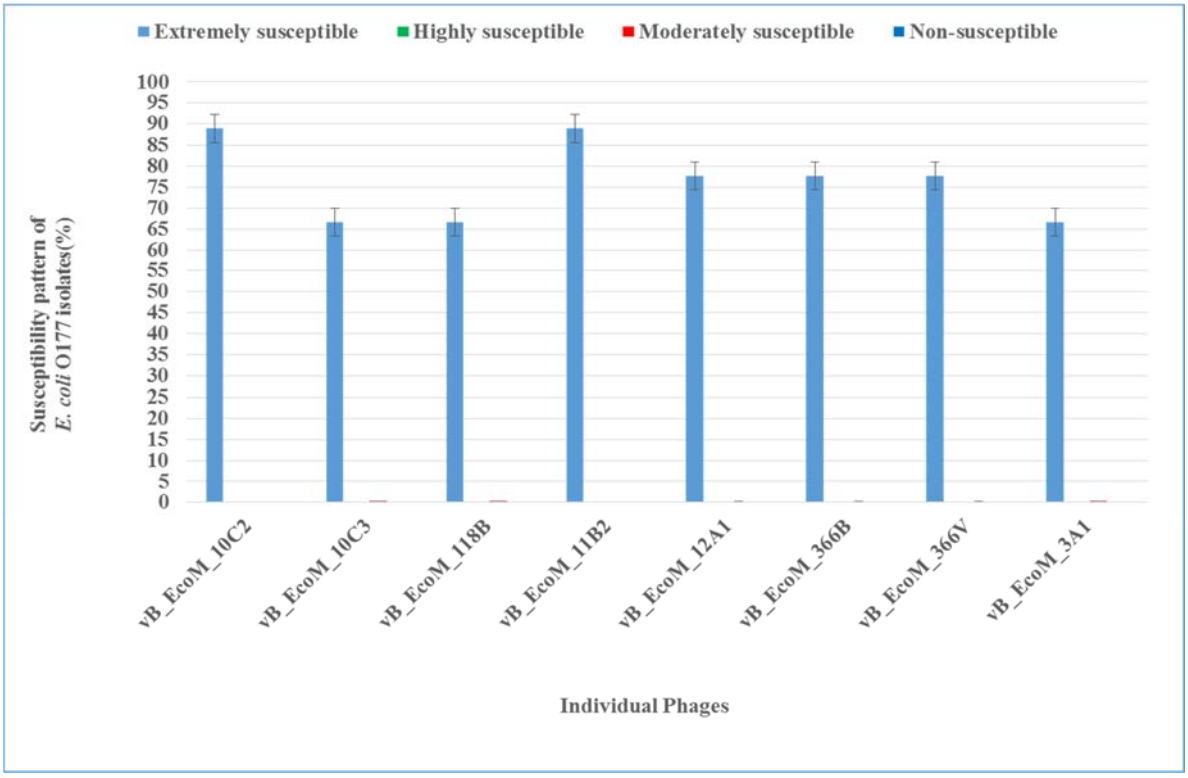

Figure 6.1: Susceptibility pattern of *E. coli* O177 isolates against individual phages. The error bars represent the standard deviation.

Table 6.1: The sensitivity of *E. coli* O177 strains based on Multiplicity of infections.

Bacteria ID	MOIs of the phages[a]							
	vB_EcoM_ 10C2	**vB_EcoM_ 10C3**	**vB_EcoM_ 118B**	**vB_EcoM_ 11B2**	**vB_EcoM_ 12A1**	**vB_EcoM_ 366B**	**vB_EcoM_ 366V**	**vB_EcoM _3A1**
CF-A1	1.2×10^{-3}	2.3×10^{-6}	4.1×10^{-3}	1.9×10^{-3}	4.5^{*}	3.4×10^{-5}	1.8×10^{-4}	5.2×10^{-3}
CF-D-D11	1.4×10^{-4}	4.4×10^{-6}	4.4×10^{-3}	2.9×10^{-4}	1.8×10^{-5}	2.1×10^{-6}	4.4×10^{-5}	3.8×10^{-3}
CF-H1	6.8^{*}	1.4×10^{-3}	4.1^{*}	1.3×10^{-5}	5.4×10^{-5}	1.8×10^{-3}	2×10^{-3}	5.9^{*}
CF-D-D4	3.8×10^{-3}	2.1×10^{-6}	2.7×10^{-6}	3.6×10^{-3}	2.2×10^{-3}	1.9×10^{-5}	3.3×10^{-4}	1.4×10^{-3}
CF-C29	3.5×10^{-5}	7.2^{*}	4.1^{*}	2.4×10^{-4}	3.4×10^{-4}	4.6×10^{-3}	3.4×10^{-4}	3.2×10^{-6}
CF-D-D32	1.3×10^{-7}	6.4^{*}	1.9×10^{-5}	1.1×10^{-3}	4.4×10^{-3}	1.4×10^{-3}	5.2^{*}	4.2×10^{-6}
CF-C1	1.9×10^{-4}	5.1×10^{-3}	4.4×10^{-3}	2.3×10^{-3}	5.1×10^{-7}	7.1^{*}	3.3×10^{-3}	7.3^{*}
CF-A15	1.6×10^{-3}	1.7×10^{-3}	6.1^{*}	4.2×10^{-4}	5.5^{*}	5.4×10^{-3}	4.6	2.8×10^{-5}
CF-E17	4.5×10^{-3}	6.6^{*}	3.1×10^{-3}	5.6^{*}	2.5×10^{-6}	4.7^{*}	1.7×10^{-3}	6.1^{*}

***Key*:** ID = Identity; superscript"**a**"denotes multiplicity of infection (the lowest ration of the phage to bacteria resulting in complete lysis of overnight culture bacteria culture within 5 hours of incubation). Asterisk (*) denotes incomplete lysis.

6.3.2. Susceptibility of *E. coli* O177 strain against phage cocktails

Thirty-two phage cocktails containing different combinations were successfully designed and susceptibility of *E. coli* O177 isolates to phage cocktails was assessed using microplate virulence assay. *E. coli* O177 isolates were extremely sensitive to all the tested phage cocktails. Most of (18) phage cocktails were highly active at low titer (from 1×10^4 to 1×10^1 PFU/mL) while 14 were active at high titer (1×10^8 PFU/mL). Based on MOI, the isolates were classified as either extremely, highly and moderately susceptible ((MOI = < 0.01, 0.01 ≤ MOI < 1 and 1 ≤ MOI < 10, respectively) to two-phage cocktails (Table 1A) or extremely and highly susceptible to three and more-phage cocktails (Table 1B – C). All three *E. coli* O177 isolates tested were sensitive to 32 phage cocktails. *E. coli* O177 isolates showed extremely susceptible to 31/32 phage cocktails, highly and moderately susceptible to 29/32 and 5/32 phage cocktails, respectively. Isolate CF-H246 further showed moderately susceptibility to four phage cocktails with their MOI ranging between 1.4 and 1.5 while isolates CF-A27 and CF-G202 were moderately susceptible to phage cocktail vB_EcoM_118B_ vB_EcoM_3A1 and vB_EcoM_10C2_ vB_EcoM_12A1 (MOI = 2.8 and 1.8, respectively). Phage cocktails, vB_EcoM_10C2_ vB_EcoM_366B and vB_EcoM_11B_ vB_EcoM_118B_EC12A1, had the lowest MOI values (average MOI = 0.00001615 and 0.000701, respectively).

Table 6.2A: Susceptibility of *E. coli* O177 strain to two-phage cocktails based on multiplicity of infections.

Phage cocktails (ID)	Host Bacteria (ID) used		
	CF-A27	CF-G202	CF-H246
	Phage cocktails MOIs		
vB_EcoM_11B2_ vB_EcoM_118B	2.3×10^{-3}	2.9×10^{-7}	1.7×10^{-3}
vB_EcoM_11B_ vB_EcoM_10C2	2.0×10^{-6}	2.6×10^{-2}	1.5^{*}
vB_EcoM_11B_ vB_EcoM_12A1	1.8×10^{-6}	2.3×10^{-2}	1.4^{*}
vB_EcoM_11B_ vB_EcoM_366B	1.9×10^{-4}	2.4×10^{-5}	1.4×10^{-2}
vB_EcoM_11B_ vB_EcoM_3A1	2.7×10^{-3}	3.5×10^{-6}	2.1×10^{-1}
vB_EcoM_118B_ vB_EcoM_10C2	4.1×10^{-5}	5.3×10^{-7}	3.1×10^{-2}
vB_EcoM_118B_ vB_EcoM_12A1	2.2×10^{-6}	2.5×10^{-7}	1.4×10^{-2}
vB_EcoM_118B_ vB_EcoM_366B	2.8×10^{-3}	3.6×10^{-7}	2.1×10^{-3}
vB_EcoM_366B_ vB_EcoM_3A1	1.4×10^{-4}	1.8×10^{-5}	1.0×10^{-1}
vB_EcoM_118B_ vB_EcoM_3A1	2.8^{*}	2.1×10^{-7}	1.2×10^{-1}
vB_EcoM_10C2_ vB_EcoM_12A1	1.4×10^{-6}	1.8^{*}	1.3^{*}
vB_EcoM_10C2_ vB_EcoM_366B	2.3×10^{-6}	3×10^{-5}	1.5^{*}
vB_EcoM_10C2_ vB_EcoM_3A1	1.7×10^{-5}	2.2×10^{-5}	1.3×10^{-1}
vB_EcoM_12A1_ vB_EcoM_366B	1.6×10^{-7}	2.1×10^{-5}	1.2×10^{-1}
vB_EcoM_12A1_ vB_EcoM_3A1	1.8×10^{-6}	2.4×10^{-5}	1.4×10^{-1}
vB_EcoM_366B_ vB_EcoM_3A1	1.4×10^{-4}	1.8×10^{-5}	1.0×10^{-1}

Table 6.2B: Susceptibility of *E. coli* O177 strain to three-phage cocktails based on multiplicity of infections.

Phage cocktails (ID)	**Host Bacteria (ID) used**		
	CF-A27	**CF-G202**	**CF-H246**
	Phage cocktails MOIs		
vB_EcoM_11B_ vB_EcoM_118B_ vB_EcoM_10C2	4.3×10^{-5}	5.6×10^{-7}	3.3×10^{-3}
vB_EcoM_11B_ vB_EcoM_118B_ vB_EcoM_12A1	2.8×10^{-6}	3.6×10^{-7}	2.1×10^{-3}
vB_EcoM_11B_ vB_EcoM_118B_ vB_EcoM_366B	2.5×10^{-3}	3.3×10^{-7}	1.9×10^{-3}
vB_EcoM_11B_ vB_EcoM_118B_ vB_EcoM_3A1	3.3×10^{-3}	4.4×10^{-7}	2.6×10^{-3}
vB_EcoM_118B_ vB_EcoM_10C2_ vB_EcoM_12A1	2.5×10^{-6}	3.3×10^{-7}	1.9×10^{-2}
vB_EcoM_118B_ vB_EcoM_366B_ vB_EcoM_3A1	1.9×10^{-3}	2.5×10^{-6}	1.5×10^{-3}
vB_EcoM_10C2_ vB_EcoM_12A1_ vB_EcoM_366B	2.2×10^{-7}	2.9×10^{-5}	1.7×10^{-1}
vB_EcoM_10C2_ vB_EcoM_366B _ vB_EcoM_3A1	2.4×10^{-6}	3.2×10^{-5}	1.9×10^{-1}

Table 6.2C: Susceptibility of *E. coli* O177 strain to four- and more-phage cocktails based on multiplicity of infections.

Phage cocktails (ID)	**Host Bacteria (ID) used**		
	CF-A27	**CF-G202**	**CF-H246**
	Phage cocktails MOIs		
vB_EcoM_11B_ vB_EcoM_118B_ vB_EcoM_10C2_ vB_EcoM_12A1	1.7×10^{-6}	2.2×10^{-6}	1.3×10^{-2}
vB_EcoM_11B_ vB_EcoM_118B_ vB_EcoM_10C2_ vB_EcoM_366B	2.9×10^{-6}	3.7×10^{-6}	2.2×10^{-2}
vB_EcoM_11B_ vB_EcoM_118B_ vB_EcoM_10C2_ vB_EcoM_3A1	3.2×10^{-5}	4.2×10^{-6}	2.4×10^{-2}
vB_EcoM_118B_ vB_EcoM_10C2_ vB_EcoM_12A1_ vB_EcoM_366B	3.8×10^{-5}	5×10^{-6}	2.9×10^{-2}
vB_EcoM_118B_ vB_EcoM_10C2_ vB_EcoM_12A1_ vB_EcoM_3A1	1.9×10^{-5}	2.5×10^{-6}	1.5×10^{-2}
vB_EcoM_10C2_ vB_EcoM_12A1_ vB_EcoM_366B_ vB_EcoM_3A1	3×10^{-5}	3.9×10^{-6}	2.3×10^{-1}
vB_EcoM_11B_ vB_EcoM_118B_ vB_EcoM_10C2_ vB_EcoM_12A1_ vB_EcoM_366B	4×10^{-5}	5.2×10^{-7}	3.1×10^{-2}
vB_EcoM_11B_ vB_EcoM_118B_ vB_EcoM_10C2_ vB_EcoM_12A1_ vB_EcoM_3A1	4.5×10^{-6}	5.9×10^{-6}	3.4×10^{-2}
vB_EcoM_11B_ vB_EcoM_118B_ vB_EcoM_10C2_ vB_EcoM_12A1_ vB_EcoM_366B_ vB_EcoM_3A1	2×10^{-6}	2.6×10^{-5}	1.6×10^{-2}

***Key*:** ID = Identity; superscript"**a**" denotes multiplicity of infection (the lowest ratio of the phage to bacteria resulting in complete lysis of overnight culture bacteria culture within 5 hours of incubation). Asterisk (*****) denotes incomplete lysis.

6.3.3. Effect of surface application of individual phages in reducing *E. coli* O177 on beef at 4 °C

The efficacy of individual phages in reducing *E. coli* O177 on artificially contaminated beef incubated at 4 °C over 0, 1, 3 and 7-day periods was assessed. Figure 6.2A illustrates the number of viable cells remaining on artificially contaminated beef after treatment with individual phages. Repeated measures analysis showed significant interaction effect between individual phage and incubation time on viable *E. coli* O177 cell counts. At day 1, phages significantly ($p \leq 0.001$) reduced *E. coli* O177 cell counts as compared to their respective day 0 counts as well as the negative control counts. Bacterial reduction ranged from 5.66 to 7.81 $\log_{10}$ CFU/mL when treated with individual phages. Relative reduction of bacterial counts ranged between 73 and 99%, Figure 6.2B. Phages vB_EcoM_118, vB_EcoM_11B, vB_EcoM_366B and vB_EcoM_366V were able to reduce bacteria to non-detection levels (1.0 log 10 CFU/mL) at day one whereas phages vB_EcoM_12A1 and vB_EcoM_3A1 were able to reduce bacteria to non-detection levels over 7-days period. At day three and seven, bacterial reduction ranged from 4.39 to 5.8 $\log_{10}$ CFU/mL and 2.1 to 4.28 $\log_{10}$ CFU/mL, respectively. A decrease in relative reduction percentage was observed and this was indicating bacterial cell count increase.

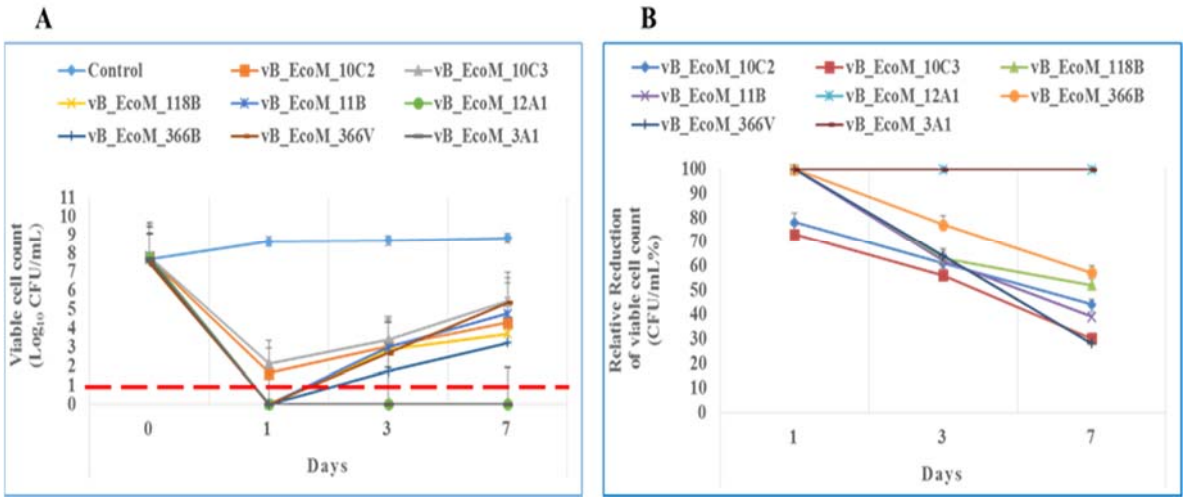

Figure 6.2: The number of *E. coli* O177 cells remaining on contaminated beef after treatment with individual phages over a 7-day period (A). Dotted red line indicate detection limit for direct plating. Relative reduction percentage of *E. coli* O177 cells after treatment with phage over a 7-day period (B). The error bars represent the standard deviation.

6.3.4. Effect of surface application of phage cocktails in reducing *E. coli* O177 on beef at 4 °C

The efficacy of phage cocktails in reducing *E. coli* O177 on artificially contaminated beef incubated at 4 °C over 7-day period was evaluated. Figure 6.3A shows the number of viable cells remaining on artificially contaminated beef after treatment with phage cocktails. Repeated measures analysis revealed significant interaction effect between phage cocktails and time of incubation on viable *E. coli* O177 cell counts on experimentally contaminated beef. At day 1, 3, and 7, phage cocktails exhibited significant reduction of viable *E. coli* O177 cell counts on beef. At day 1, relative reduction of bacterial counts ranged between 32 and 99.99%, Figure 6.3B.

Phage cocktails T3, T4 and T6 exhibited the highest (below detection limit) reduction of viable *E. coli O177* cell counts on contaminated beef over 7-day period, Figure 6.3A. At day 1, phage cocktails T1, T2 and T5 showed significant ($p \leq 0.001$) reduction on bacterial cell count 2.36, 2.07 and 1.87 $\log_{10}$ CFU/mL, respectively) as compared to their respective day 0 counts. At

days 3 and 7, the reduction of viable *E. coli* O177 cell counts ranged from 1.98 to 1.83 log_{10} CFU/mL and 0.94 to 1.2 log_{10} CFU/mL, respectively. A decrease in relative reduction percentage of bacteria cell count was observed at days 3 and 7, Figure 4B (T1, T2 and T5). Despite this, phages significantly ($P < 0.001$) reduced viable *E. coli* O177 cell counts compared to control (meat treated with lambda diluent alone).

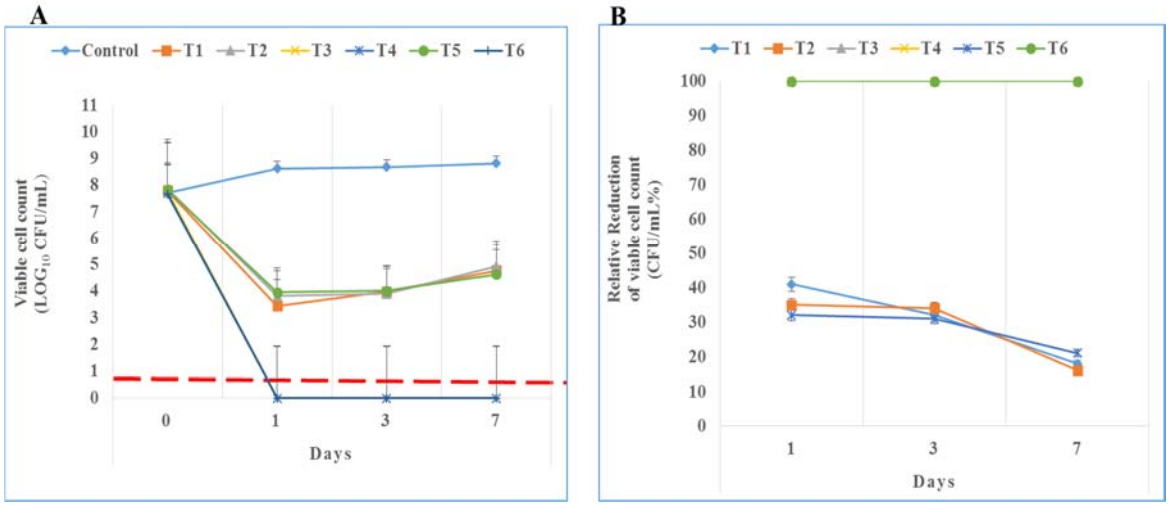

Figure 6.3: The number of *E. coli* O177 cells remaining on contaminated beef after treatment with phage cocktails over a 7-day period (A). Dotted red line indicate detection limit for direct plating. Relative reduction percentage of *E. coli* O177 cell count after treatment with phage cocktails over a 7-day period (B). The error bars represent the standard deviation.

6.3.5. Biofilm formation by *E. coli* O177

Biofilm assay was performed to assess the ability of *E. coli* O177 to form biofilms when incubated at 37 °C and 25 °C for 24 and 48 hours. The results revealed that *E. coli* O177 was able to form biofilm on polystyrene plate surface when incubated at different temperatures, Figure 6.4A - B. Strong biofilm formation was relatively high at 25 °C (22.2%) than 37 °C (11.1%) after 24-hour incubation period ($P < 0.05$). Similarly, after 48 hours incubation period, 29.6% and 17.3% of the isolates produced strong biofilm (25 °C and 37 °C, respectively). At 25 °C and 24 hours of incubation, 2.5% of isolates were classified as weak and 9.9% as

moderate producers while at 37 °C, 7.4% of isolates were classified as weak and 8.6% moderate producers. At 25 °C and 48 hours of incubation 3.7% isolates were classified as weak producers and (4.9%) moderate producers whereas at 37 °C isolates were classified as weak (7.4%) and moderate (13.6%) biofilm producers.

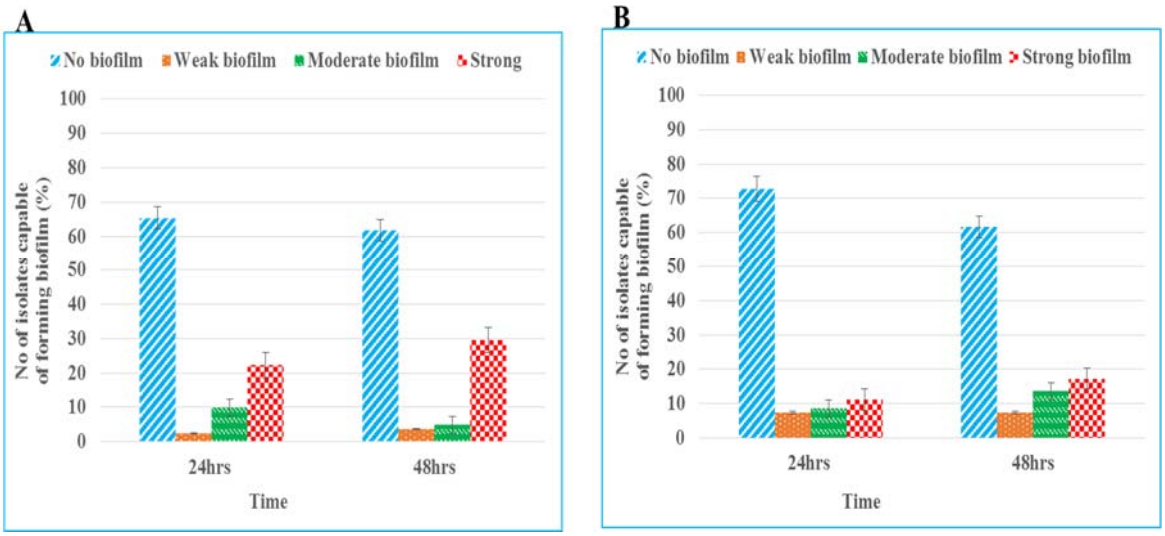

Figure 6.4: Biofilm formation by *E. coli* O177 isolates on 96-well polystyrene plates. (**A**) Biofilm formation by 81 *E. coli* O177 isolates when incubated at 25 °C for 24 and 48 hours. (**B**) Biofilm formation by 81 *E. coli* O177 isolates when incubated at 37 °C for 24 and 48 hours. The bars indicate standard deviation. Cut-off values 0.10 and 0.14 at OD_{600nm} (for 24 hours and 48 hours, respectively) were used to classify the isolates as OD ≤ ODc, no biofilm formation; ODc < OD ≤ 2 x ODc, weak biofilm formation; 2 x ODc < OD ≤ 4 x ODc, moderate biofilm formation; 4 x ODc < OD, strong biofilm formation.

6.3.6. Efficacy of individual phages and phage cocktails in preventing biofilm formation by *E. coli* O177

The ability of individual phages and cocktails in preventing biofilm formation was evaluated against 10 *E. coli* O177 (strong biofilm producers) isolates. As shown in Figure 6.5, individual phages significantly ($P < 0.05$) prevented biofilm formation. All individual phages were able to inhibit strong biofilm formation by *E. coli* O177. Most of the isolates (8/10) did not form biofilm in the presence of phages vB_EcoM_10C3, vB_EcoM_10C2 and vB_EcoM_366V at 25 °C for 24-hour incubation period while a small number of isolates were able to form weak and moderate biofilm in the presence of the phages. Phage cocktails revealed high capabilities of preventing biofilm formation by *E. coli* O177 isolates incubated at 25 °C for 24 hours, Figure 6.6. No *E. coli* O177 isolates formed biofilm in the presence of phage cocktails. No weak, moderate and/or strong biofilm producers were observed when treated with phage cocktails.

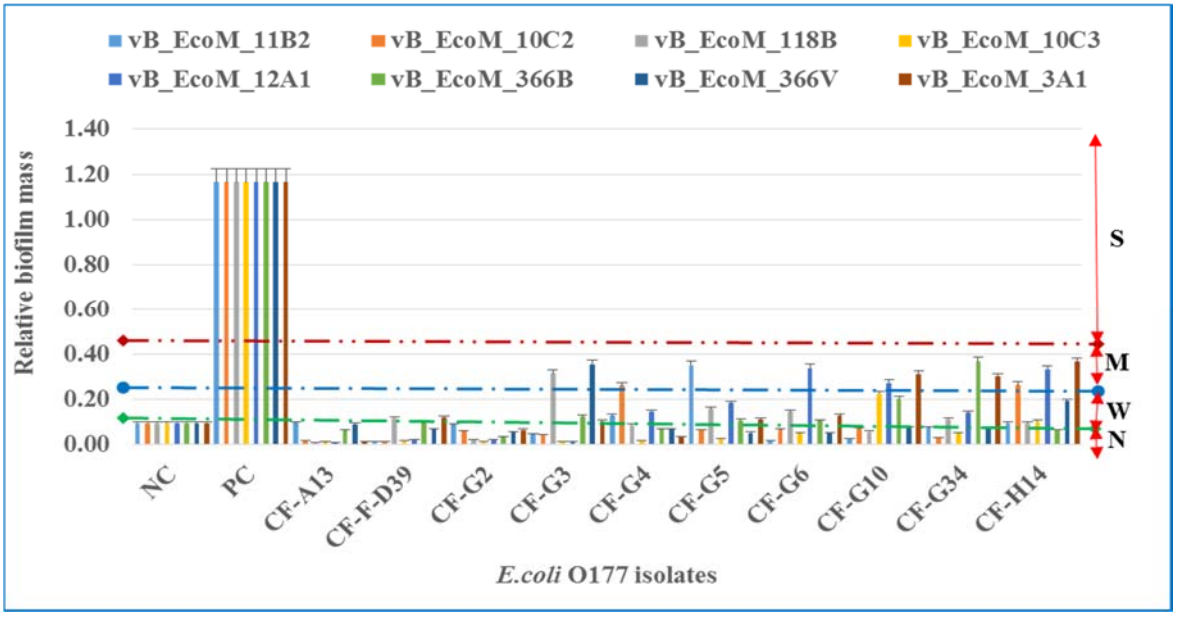

Figure 6.5: Efficacy of individual phages in reducing biofilm formation by *E. coli* O177 strain at 25 °C for 24-hour incubation period. The Y-axis represent the optical density of each isolates determined at OD_{600nm} while the X-axis represent *E. coli* O177 isolates. ***Key*: S** = strong (4 x ODc < OD); **M** = moderate (2 x ODc < OD ≤ 4 x ODc); **W** = weak (ODc < OD ≤ 2 x ODc); N = no biofilm formation (OD ≤ ODc); NC= negative control; PC = positive control. A cut-off

value 0.10 at OD_{600nm} was used to classify the isolates. The error bars represent the standard deviation.

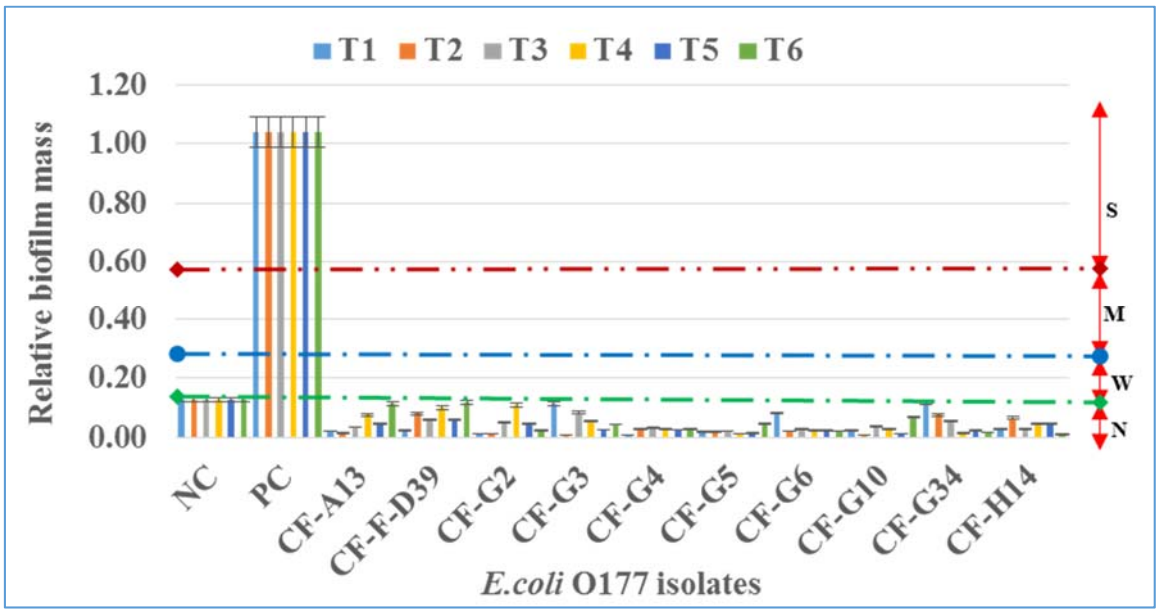

Figure 6.6: Efficacy of phage cocktails in preventing biofilm formation by *E. coli* O177 strain at 25 °C for 24-hour incubation period. The Y-axis represent the optical density of each isolates determined at OD_{600nm} while the X-axis represent *E. coli* O177 isolates. ***Key*: S** = strong (4 x ODc < OD); **M** = moderate (2 x ODc < OD ≤ 4 x ODc); **W** = weak (ODc < OD ≤ 2 x ODc); N= no biofilm formation (OD ≤ ODc); **NC** = negative control; **PC** = positive control. A cut-off value 0.13 at OD_{600nm} was used to classify the isolates. The error bars represent the standard deviation. The error bars represent the standard deviation.

6.3.7. Effect of individual phages in destroying formed biofilm mass by *E. coli* O177

The potential of individual phages to destruct biofilm mass formed by 10 *E. coli* O177 isolates was evaluated at 25 °C for a period of 5 hours. Individual phages revealed various patterns in disintegrating established biofilm structure, Figure 6.7. Generally, all individual phages revealed significant ($P < 0.05$) destruction of biofilm mass formed by CF-A13 and CF-G10 isolates to non-biofilm producer category (OD ≤ ODc). Five individual phages were able to disintegrate biofilm mass formed by CF-H14 isolate to weak category. All the phages revealed similar destruction patterns on biofilm mass formed by CF-D39, CF-G2 and CF-G3 isolates

and their biofilm mass were classified under moderate category. However, phages were not able to destruct the biofilm mass of four isolates (CF-G4, CF-G5, CF-G6 and CF-G34), Figure 6.7.

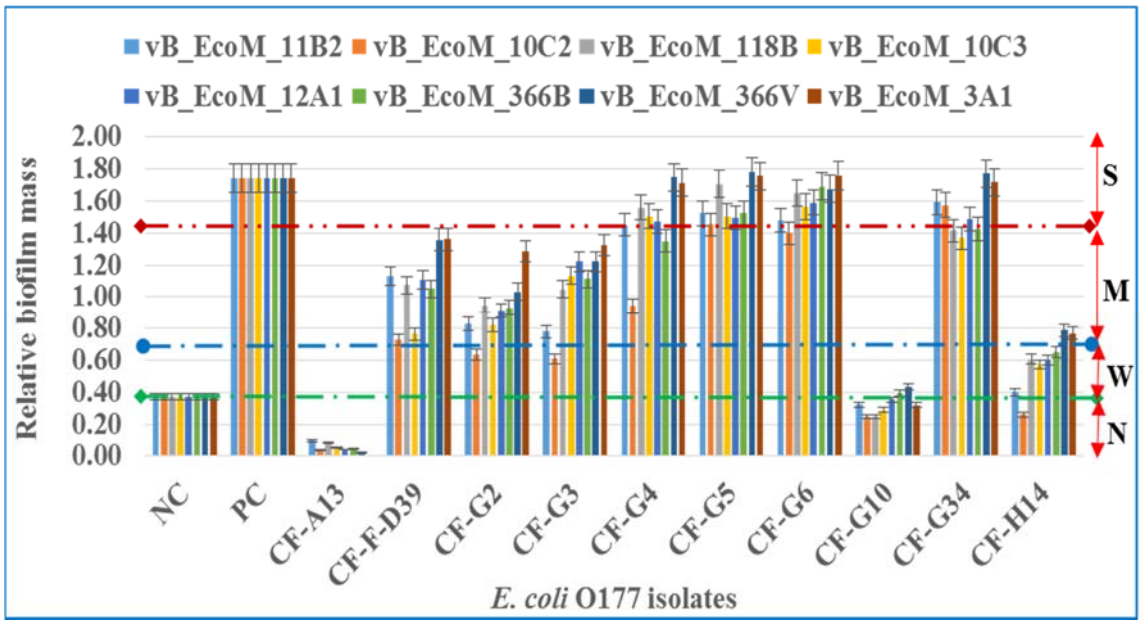

Figure 6.7: Efficacy of individual phages in destroying pre- formed biofilm by *E. coli* O177 strain at 25 °C for 5-hour incubation period. The Y-axis represent the optical density of each isolate determined at OD_{600nm} while the X-axis represent *E. coli* O177 isolates. The error bars represent the standard deviation. ***Key*: S** = strong (4 x ODc < OD); **M** = moderate (2 x ODc < OD ≤ 4 x ODc); **W** = weak (ODc < OD ≤ 2 x ODc); **N** = no biofilm formation (OD ≤ ODc); **NC** = negative control; **PC** = positive control. A cut-off value 0.37 at OD_{600nm} was used to classify the isolates. The error bars represent the standard deviation.

6.3.8. Effect of phage cocktails in destroying formed biofilm mass by *E. coli* O177

The ability of phage cocktails to destruct biofilm mass formed by 10 *E. coli* O177 isolates was assessed at 25 °C for a period of 5 hours. Phage cocktails revealed efficacy in disintegrating established biofilm mass formed by *E. coli* O177 isolates, Figure 6.8. Phage cocktails were able to destruct biofilm mass to no, weak and moderate categories. All phage cocktails were able to disintegrate biofilm mass of 5 (50%) and 4 (40%) isolates to no and weak biofilm

producer categories, respectively. Isolate CF-G34 biofilm mass was classified as moderate against one phage cocktail (T6).

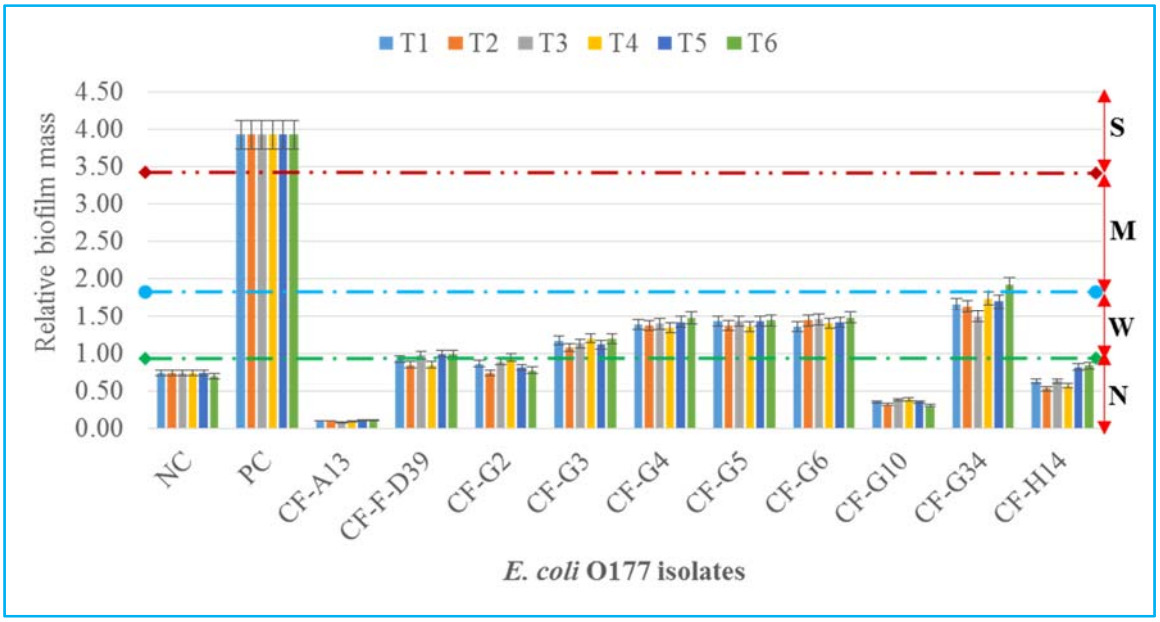

Figure 6.8: Efficacy of phage cocktails in destroying pre- formed biofilm by *E. coli* O177 strain at 25 °C for 5-hour incubation period. The Y-axis represent the optical density of each isolate determined at OD_{600nm} while the X-axis represent *E. coli* O177 isolates. The error bars represent the standard deviation. ***Key*: S** = strong (4 x ODc < OD); **M** = moderate (2 x ODc < OD ≤ 4xODc); **W**= weak (ODc < OD ≤ 2 x ODc); **N** = no biofilm formation (OD ≤ ODc); **NC** = negative control; **PC** = positive control. A cut-off value 0.83 at OD_{600nm} was used to classify the isolates. The error bars represent the standard deviation.

6.4. Discussion

Lytic capabilities and multiplicity of infection are the most critical parameters to be evaluated when selecting phage candidates for developing phage cocktails (Niu *et al.*, 2009; Stratakos and Grant, 2018). In this study, microplate virulence assay was performed to evaluate lytic capabilities of individual phages and cocktails against a panel of nine (9) multi-drug resistant *E. coli* O177 isolates obtained from cattle faeces. Microplate-based approach provides information on the phage virulence, MOI and the possibilities of phage resistance (El-

Dougdoug *et al.*, 2019). Individual phages revealed various lytic patterns against the *E. coli* O177 isolates tested. All the *E. coli* O177 isolates tested were highly susceptible to all the phages. These results are similarly to those reported by the previous studies (Niu *et al.*, 2009). Sensitivity pattern ranged from 67 to 89%. Phage vB_EcoM_10C2 and vB_EcoM_11B2 revealed the highest efficacy against the host. This could be attributed to the presence of the common receptors such as lipopolysaccharides and O-antigen on host bacterial cell wall (Niu *et al.*, 2009). Based on MOI, all the isolates were classified as extremely to moderately susceptible against each individual phage. However, incomplete lysis of the bacteria against each phage was observed, and this could be due to phages resistant mutants (Chan *et al.*, 2013).

Thirty-two (32) phage cocktails were successfully designed and assessed for their lytic activities against three *E. coli* O177 isolates. Optimized phage cocktails revealed high lytic capabilities against all the isolates tested. Interestingly, phage cocktails displayed high efficacy at low titer (1×10^1 PFU/mL) concentration as compared to their respective individual phages. Based on the MOI, phage cocktails made up of a combination of two to five individual phages displayed the highest lytic capabilities against *E. coli* O177 isolates. However, cocktails made up of six and more phages revealed similar results with five-phage cocktails. This indicated that increasing the number of individual phages (six and more phages) in cocktail did not improve lytic capabilities in this study. A similar observation was reported by the previous studies (Chen *et al*., 2018; Duc *et al.*, 2018). This could be attributed to the fact that high phage cocktail titers tend to aggregate and thus attenuate phage activity. In addition, competitive interaction for a common receptor among the phages of the same or different families may reduce their efficacy (Liu *et al.*, 2015; Chen *et al.*, 2018). Unlike in individual phages, the incidence of incomplete lysis of the bacteria was very low with cocktails. These findings suggest that phage cocktails designed in this study have potential in killing host bacteria.

Numerous studies have demonstrated the efficacy of either individual phages or phage cocktails in reducing foodborne pathogens on experimentally contaminated vegetables and meat (Hungaro *et al.*, 2013; Tomat *et al.*, 2014; Pereira *et al.*, 2016; Yeh *et al.*, 2017; Duc *et al.*, 2018; Huang *et al.*, 2018). In this study, treatment of artificially contaminated beef with both the individual phages and cocktails revealed significant reduction of viable *E. coli* O177 cell counts over the seven-day period. All phages (both individual phages and cocktails) displayed highest reduction of bacterial cell counts within 24 hours of incubation period. Six individual phages and three phage cocktails reduced bacterial cell count below detection limit (1.0 $\log_{10}$ CFU/mL) on day one. Interestingly, two individual phages and three cocktails showed progressive reduction of viable cell count below detection limit over a seven day-incubation period. In addition, the relative reduction of cell count ranged from 78 to 100% (individual phages) and 32 to 100% (phage cocktails) at day 1. This showed that most of individual phages were highly active in reducing bacteria cell count at day 1. Even though bacterial cell count showed increase on meat treated with individual phages and cocktails was observed at day 3 and 7, viable cell count was significantly lower than the initial inoculum cell count and their respective controls. Similar observation was reported in other studies, where bacteria showed regrowth after 24 hours exposure to phages (Wang *et al.*, 2017; Huang *et al.*, 2018; Tomata *et al.*, 2018; Bai *et al.*, 2019). Bacterial regrowth could be due to complexity of beef matrix interfering with diffusion and distribution of the phages and thus decreasing the likelihood of phage-host interaction (Tomat *et al.*, 2014; Liu *et al.*, 2015; Duc *et al.*, 2018). Furthermore, regrowth could be because of phage resistant mutants.

Another concern relating to food safety is biofilm formation by food-spoiling agents. Biofilms are common in food industry and they cause food contamination and corrosion on food processing plants (Carpio *et al.*, 2019). Moreover, a biofilm provides a protective mechanism

to bacteria because it is resistant to conventional disinfectant agents (Merino *et al.*, 2019; Carpio *et al.*, 2019). Lytic phages may serve as an alternative to inhibit and destroy biofilm in food. The current study assessed the ability of *E. coli* O177 to form biofilm at different temperatures for the very first time. It was observed that *E. coli* O177 is capable of forming biofilm on polystyrene surface at different temperatures (25 °C and37 °C). Biofilm formation was frequently detected at 25 °C compared to 37 °C. Even though some studies reported biofilm formation at low temperatures (4 °C, 10 °C and 15 °C) (Han *et al.*, 2016; Sadekuzzaman *et al.*, 2017;), a pilot study conducted prior to the actual experiment revealed that *E. coli* O177 did not form biofilm at 4 °C. This indicates that biofilm formation depends on the nature of bacteria species and environmental temperature.

The ability of individual phages and phage cocktails in preventing biofilm formation by *E. coli* O177 (strong biofilm producers) strain was assessed using microplate-based assay. Individual phages and cocktails inhibited biofilm formation at 25 °C over a 24-hour incubation period. When treated with individual phages, no isolates formed strong biofilm. Only weak and moderate biofilm formations were observed after 24 hours of the incubation period. On the other hand, phage cocktails were more effective in preventing biofilm formation. Of all the isolates tested, no isolate was able to form biofilm in the presence of phage cocktails. This clearly demonstrate that phage cocktails used in this study are highly effective in preventing biofilm formation by *E. coli* O177. Unlike other studies, which reported similar results after 48 to 144 hours of incubation period (Endersen *et al.*, 2017; Sadekuzzaman *et al.*, 2017), this study reports the ability of both individual phages and cocktails in preventing biofilm formation after 24 hours incubation period.

Another important observation was that individual phages and cocktails were able to destroy established biofilm structure. Individual phages revealed various patterns in destructing strong biofilm mass formed by *E. coli* O177 at 25 °C over a 5-hour period. Interestingly, all individual phages were able to destruct biofilm masses of two of the tested isolates to non-biofilm producer category. However, individual phages were not able destruct the biofilm masses of four isolates. This demonstrates that individual phages were more effective in inactivating the planktonic cells than destroying pre-formed biofilms. On the other hand, phage cocktails revealed more effective in destructing biofilms and no strong biofilm producer category was observed. Similar results were reported in one of the previous study, which evaluated the efficacy of lytic *Pseudomonas aeruginosa*-specific phages in destroying established biofilms (Yuan *et al.*, 2019).

6.5. Conclusion

This study revealed that both individual phages and phage cocktails are capable of reducing *E. coli* O177 viable cells on raw beef. Individual phages and cocktails were also capable of preventing biofilm formation. Furthermore, phage cocktails reduced pro-formed biofilm mass to weak and non-biofilm category. This clearly showed that phage cocktails have potential as a stand-alone treatment for the control biofilm formation and reduction of *E. coli* cells in beef, and possibly in other food items.

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CHAPTER SEVEN

VIABILITY OF PHAGES AND THEIR POTENCY AGAINST *ESCHERICHIA COLI* O177 STRAIN IN A SIMULATED RUMEN FERMENTATION SYSTEM

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VIABILITY OF PHAGES AND THEIR POTENCY AGAINST *ESCHERICHIA COLI* O177 IN A SIMULATED RUMEN FERMENTATION SYSTEM

Abstract

The control of *Escherichia coli* in food producing animals has become a major challenge in the food industry. Phage therapy is considered as a viable alternative strategy for biocontrol of this pathogen in live ruminants. The extent to which *E. coli* colonises the rumen in live cattle has a direct effect on the risk of contamination of meat products at slaughter. Despite this, there are no scientific studies investigating the effectiveness of phages in reducing *E. coli* O177 in the rumen. Therefore, the objective of this study was to assess the viability and effectiveness of individual phages and their cocktails in reducing *E. coli* O177 cell counts in a simulated ruminal fermentation system over a 48-hour incubation period. Eighty serum bottles containing rumen fluid obtained from a Bonsmara cow were randomly assigned to the sixteen phage treatments [eight individual phages (*E. coli* O177 and phage added), six phage cocktails (*E. coli* O177 and phage added), one negative control (*E. coli* O177 without phages) and one positive control (neither *E. coli* O177 nor phage added)]. For phages, *E. coli* O177 enumeration, and pH measurements, samples were taken at 0, 6, 12, 24, 36, 48-hour points.

Response surface regression analysis showed significant quadratic responses in the titres of both individual phages and their cocktails over the 48-hour incubation period under simulated rumen fermentation conditions (absence of oxygen, 39 °C, and a pH range of 6.6 - 6.8). Individual phage titres were predicted to peak at 50 - 52 hours of *in vitro* ruminal incubation from response surface regression models with R^2 values ranging from 0.811 to 0.994. On the

other hand, phage cocktail titres were predicted to peak at 51 and 55 hours of *in vitro* ruminal incubation from response surface regression models with R^2 values ranging from 0.982 to 0.995. Furthermore, percent reduction of *E. coli* O177 cell counts upon exposure to individual phages and phage cocktails followed a quadratic response over time.

Response surface regression analysis also revealed significant quadratic trends in percentage reduction of *E. coli* O177 cell counts when exposed to individual phages and their cocktails over the 48-hour incubation. When treated with individual phages, percentage reduction of *E. coli* O177 cell counts peaked (60.81 - 63.27%) at 47 to 48 hours of incubation as determined from prediction equations with R^2 values ranging from 0.992 to 0.996. However, when exposed to phage cocktails, percentage reduction of *E. coli* O177 cell counts peaked (63.06 to 73.25%) at 43 to 46 hours of incubation determined from prediction equations with R^2 values ranging from 0.970 to 0.993. Over the 48-hour of incubation period, individual phages reduced *E. coli* O177 cell counts by between 16.03 and 62.74%. Individual phages vB_EcoM_366B and vB_EcoM_3A1 were the most effective (62.31 and 62.74%, respectively) phages while vB_EcoM_10C2 and vB_EcoM_10C3 were the least effective (61.02 and 61.12%) phages in reducing *E. coli* O177 cell count at 39 °C over 48 hour incubation period. On the other hand, phage cocktails reduced *E. coli* O177 cell counts by between 20.65 and 66.92%. Phage cocktails T1, T3, T4 were the most effective (66.67, 66.92 and 66.42%, respectively) while T2 and T5 were the least effective (62.82 and 62.35%, respectively) in reducing *E. coli* O177 cell count at 39 °C over 48 hour incubation period. These results indicate that phage cocktails T3, T4, and T6 are the most effective in reducing *E. coli* O177 cells counts in a simulated ruminal fermentation system. Therefore, these phage cocktails are the most suitable candidates to be used in live animals, particularly cattle to reduce the level of *E. coli* O177 load before slaughter.

Keywords: *E. coli* O177, rumen simulation, cattle, phage cocktail, biocontrol

7.1. Introduction

Atypical enteropathogenic *E. coli* (aEPEC) strains cause severe diarrhoea in humans, particularly in both developed and developing countries (Ingle *et al.*, 2016). The aEPEC strains lack *bfp* operon, which differentiates them from the other pathogenic *E. coli* strain groups (Trabulsi *et al.*, 2002; Kaper *et al.*, 2004). In addition, aEPEC such as *E. coli* O177 strain harbour *stx*, *hlyA* and *eaeA* virulence genes (Álvarez-Suárez *et al.*, 2016; Montso *et al.*, 2019). Furthermore, aEPEC strains are commonly found in the gastrointestinal tracts (GIT) of animals, especially ruminants (Trabulsi *et al.*, 2002; Martins *et al.*, 2016; Montso *et al.*, 2019). Cattle may spread these pathogens via faecal shedding and thus contaminate environments, water and animal products such as meat and milk (Ateba and Mbewe, 2011; Álvarez-Suárez *et al.*, 2016). Furthermore, contaminated hides have been associated with meat contamination during milking and slaughtering (Goodridge and Bisha, 2011; Brauer *et al.*, 2019). As a result, contaminated animal products may disseminate foodborne pathogens to humans. Current food safety interventions have serious drawbacks necessitating novel and practical interventions (Brauer *et al*., 2019). Biocontrol interventions such as phages are considered as viable alternative to control dissemination of foodborne pathogens from animals to human via food products.

Bacteriophages are highly specific to their preferred hosts and thus cause minimal distortions of the surrounding microbiome (Sillankorva *et al.*, 2012; Dissanayake *et al.*, 2019). In addition, phages are safe because they do not infect animal cells (Sillankorva *et al.*, 2012). Based on these traits, phage biocontrol is increasingly recognised as a natural and green technology, which can be applied in live animals to improve safety of animal-derived food products (Moye *et al.*, 2018). Phages selected for biocontrol purpose must be strictly lytic, stable at various pHs and temperatures while exhibiting a wide host range (Sillankorva *et al.*, 2012). In the context

of a "farm-to-fork" concept, phage biocontrol can be applied at various levels of food production to improve food safety (Sillankorva *et al.*, 2012; Endersen *et al.*, 2014). Indeed, phages have been used for the pre-harvest and/or post-harvest biocontrol of pathogens (Sillankorva *et al.*, 2012; Hussain *et al.*, 2017).

Although *E. coli* is commonly found in the lower part of the ruminant's gastrointestinal tract (Laven *et al.*, 2003), pre-slaughter starvation, subacute rumen acidosis and diets with high grain content, particularly in intensive farming systems (feedlot and dairy) may enhance proliferation of *E. coli* species in the rumen (Rasmussen *et al.*, 1993; Tkalcic *et al.*, 2000; Khafipour *et al.*, 2011). This may lead to high *E. coli* population being shed through the faeces as well as at slaughter. Consequently, food products such as milk and meat may be exposed to foodborne contamination with repercussions on human health (Mateus-Vargas *et al.*, 2017). No study has evaluated the viability of phages and their effectiveness in reducing *E. coli* O177 strain in the rumen, a possible source of the pathogen when ruminants undergo the 24-hour starvation prior to slaughter. Therefore, this study was designed to evaluate the potency and effectiveness of individual phages and phage cocktails in reducing *E. coli* O177 cells in an *in vitro* rumen fermentation.

7.2. Materials and methods

7.2.1. Grass hay substrate

Milled *Eragrotis plana* hay was analysed for dry matter (DM), organic matter (OM), crude protein (CP) content using the AOAC methods (AOAC 1999) and used as a substrate in the *in vitro* ruminal fermentation system. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined using the ANKOM2000 Fibre Analyzer (ANKOM Technology, Fairport, NY, USA) as previously described (Van Soest *et al.*, 1991). A heat stable α-amylase

was used for NDF analysis. Acid detergent lignin (ADL) was determined by treating ADF residue with 72% sulphuric acid to dissolve the cellulose. The chemical composition of the grass hay was 959.7, 918.2, 738.2, 388.3, 80.76, 60.75, and 41.5 g/kg for DM, OM, NDF, ADF, ADL, CP, and ash, respectively. The grass hay was autoclaved (at 121 °C with pressure held at 15 PSI for 15 minutes) in a 2 L beaker prior to its use as an *in vitro* ruminal fermentation substrate. After autoclaving, the grass was kept in the beaker and covered with an aluminium foil until needed for the *in vitro* fermentation experiment. Eighty sterile serum bottles were used as fermentation vessels into which 1 g of the sterile grass hay was weighed. Ninety milliliter of buffer (pH 6.8) [26 mM $NaHPO_4$ $12H_2O$, 117 mM $NaHCO_3$, 8 mM NaCl, 7.65 mM KCl, 0.63 mM $MgCl_2 \cdot 6H_2O$, 0.54 mM $CaCl_2$ $6H_2O$] were then added to each bottle. The bottles were kept in the incubator overnight at 39 °C to ensure the buffer was at the appropriate temperature at the time of rumen fluid inoculation.

7.2.2. *Escherichia coli* O177 culture

Antibiotic resistant atypical enteropathogenic *E. coli* O177 strain (isolated in Chapter 3) was used in the *in vitro* ruminal fermentation system. Briefly, a pure culture of *E. coli* O177 was removed from −80 °C freezer and resuscitated on MacConkey agar. The plates were incubated at 37 °C for 24 hours. After incubation, a single colony was transferred into 50 mL sterile falcon tube containing 10 mL nutrient broth and the tube was incubated at 37 °C for 24 hours. After incubation, the optical density (OD at 630mn) was measured using spectrophotometer. Nutrient broth was used to adjust the OD to 0.5 McFarland standard.

7.2.3. Preparation of individual phages and phage cocktails

Eight individual phages (isolated as described in Chapter 5) and six phage cocktails were evaluated in this experiment, Table 7.1. Phages were propagated using *E. coli* O177 host as previously described (Sambrook and Russell, 2001). In brief, 100 µL (0.5 McFarland standard) of overnight culture of *E. coli* O177 was added into a 50 mL falcon tubes containing 30 mL tryptic soya broth supplemented with 10 Mm magnesium sulphate (MgSO4 $7H_2O$). Aliquot of 100 µL of each phage was added to their respective tubes. The tubes were incubated at 37 °C for 24 hours in a shaking incubator (80 rpm). After incubation, the tubes were centrifuged at 10 000 × g for 10 minutes at 4 °C. The supernatant was filter-sterilised using 0.22 µm pore-size acrodisc syringe filter. Ten-fold serial dilutions were prepared and plaque assay was performed to determine the titer of each phage. The concentration was expressed as plaque forming unit per millimetre (PFU/mL). Six phage cocktails were prepared by mixing individual phages of equal volume (depending on the number of phages mixed per cocktail). The final concentration was adjusted using lambda diluent buffer (pH 7.2) to obtain the working concentration, Table 7.1.

Table 7.1: List of individual phages and phage cocktails evaluated in this experiment.

Phages	Phage ID	Adjusted titer (PFU/mL)
Individual	vB_EcoM_10C2	1 x 10^8
	vB_EcoM_10C3	1 x 10^8
	vB_EcoM_118B	1 x 10^8
	vB_EcoM_11B2	1 x 10^8
	vB_EcoM_12A1	1 x 10^8
	vB_EcoM_366B	1 x 10^8
	vB_EcoM_366V	1 x 10^8
	vB_EcoM_3A1	1 x 10^8
Cocktails	vB_EcoM_12A1_vB_EcoM_366B[a]	1 x 10^8
	vB_EcoM_10C2_vB_EcoM_12A1_vB_EcoM_366B[b]	1 x 10^8
	vB_EcoM_118B_vB_EcoM_12A1[c]	1 x 10^8
	vB_EcoM_11B2_vB_EcoM_118B_vB_EcoM_12A1[d]	1 x 10^8
	vB_EcoM_11B2_vB_EcoM_118B_vB_EcoM_366B[e]	1 x 10^8
	vB_EcoM_11B2_vB_EcoM_118B[f]	1 x 10^8

***Key*:** Superscripts "**a**, **b**, **c**, **d**, and **f**" denote T1, T2, T3, T4, T5 and T6, respectively.

7.2.4. *In vitro* ruminal fermentation

The Reading Pressure Technique (Mauricio *et al*., 1999) was used to evaluate the viability and effectiveness of individual phages and phage cocktails in reducing *E. coli* O177. Rumen fluid was obtained from a fistulated, a five-year old Bonsmara cow (body weight 650 ± 35 kg) fed on a grass hay (*Eragrotis plana*) basal diet and unlimited access to portable water. Rumen fluid was collected in the morning before feeding. The cow was placed in a crush-pen and minimally restrained. The outer area of the fistula was disinfected with cotton wool impregnated with

methylated spirit to prevent contamination. Then, the fistula was opened and the rumen contents were physically collected with sterile arm-length gloves and squeezed to strain the fluid into 2.5 L sterile thermos flask to maintain the temperature (39 °C). The thermos flask was purged with carbon dioxide (CO_2) before and after putting the fluid to create and maintain anaerobic conditions. The thermos flasks containing the fluid was immediately taken to the laboratory where the rumen contents were mixed using a sterile blender. The mixture was then strained using a sterile double-layer cheesecloth and the filtrate was transferred into a new thermos flask purged with CO_2 and held at 39 °C water bath. The serum bottles containing buffer and grass hay were purged with CO_2 and 10 mL of rumen fluid was added. The residual rumen fluid was used to measure pH and screen for the presence of *E. coli* O177-specific phages, background *E. coli* O177 strain and/or any microbial load.

7.2.5. Inoculation of *E. coli* O177 and phages

An aliquot of 200 µL overnight culture of *E. coli* O177 (1 x 10^8 CFU/mL) was added to each serum bottle except for the control (blank) bottles. An aliquot of 2000 µL of each phage treatment (individual phages and phage cocktails at 1 x 10^8 PFU/mL) was added to serum bottles at MOI of 10 (five serum bottles per phage treatment). The bottles were randomly assigned to the sixteen phage treatments [eight individual phages (*E. coli* O177 and phage added), six phage cocktails (*E. coli* O177 and phage added), one negative control (*E. coli* O177 without phages) and one positive control (neither *E. coli* O177 nor phage added)]. The bottles were purged with CO_2, sealed with rubber stoppers and incubated at 39 °C for a 48-hour period. Fermentation gases were released at 0, 6, 12, 24, 36, 48 hours during which samples were also taken and measurements carried out as described below.

7.2.6. Enumeration of bacteria, individual phages and cocktails

Fermentation medium samples were taken from each bottle at 0, 6, 12, 24, 36 and 48 hours for enumeration of bacteria, individual phages and phage cocktails. For enumeration of bacteria and phages at each time point, an aliquot of 5 mL was withdrawn from each bottle from both treated (individual phage and phage cocktails) and untreated (negative control and positive control) and 1.5 mL was transferred into 2 mL sterile eppendorf tubes. The residual samples were used to measure pH. The samples were centrifuged at 10 000 × g for 10 minutes at 4 °C to remove the phages. The supernatant was filter-sterilised using 0.22 µm pore-size acrodisc syringe filter for enumeration of individual phages and cocktails. The pellet was resuspended in 1.5 mL rumen buffer (pH 6.8). A ten-fold serial dilutions were prepared and 100 µL was plated in triplicates on MacConkey agar and the plates were incubated at 37 °C for 24 hours for viable bacterial cell count. Phage lysates (individual phages and phage cocktails) were serially diluted using rumen buffer (pH 6.8) and plaque assay was performed in triplicates to determine phage titers at each time point.

7.2.7. Statistical analysis

The data for efficacy of individual phages and phage cocktails in reducing *E. coli* in the *in vitro* ruminal fermentation medium were analysed using General Linear Model procedures of SAS (2010) version 9.3. Treatment means were separated using the least significant difference. The GLM model used was:

$$Y_{ij} = \mu + Pi + Eij,$$

where Y_{ij} is the observation of the dependent variable ij; μ is the population mean; P_i the effect of phages and E_{ij} the random error associated with ij.

Responses in phage forming units and bacterial cell counts to incubation time was analysed for linear and quadratic effects using response surface regression analysis (PROC RSREG; SAS 2010) according to the following model:

$$y = ax^2 + bx + c$$

where y = response variable, b and c are the coefficients of the quadratic equation; c is intercept; x is time (hours) and $\frac{-b}{2a}$ is the x value for optimal response. For all statistical tests, significance was declared at $P < 0.05$.

7.3. Results

7.3.1. Viability of phages and time–induced changes in total bacterial counts in a simulated rumen fermentation system

Response surface regression analysis revealed significant ($P < 0.05$) increase in titres (PFU/mL) of both individual phages and their cocktails over 48-hour incubation period. Figure 7.1A-B shows phage titre and bacteria concentration growing trend at 39 °C over 48-hour incubation period. When exposed to either individual phages or phage cocktails, bacteria concentration tended to decline while phages tended to increase, Figure 7.1A-B. This indicated that the phages were killing the bacteria hence the phage titre kept on increasing.

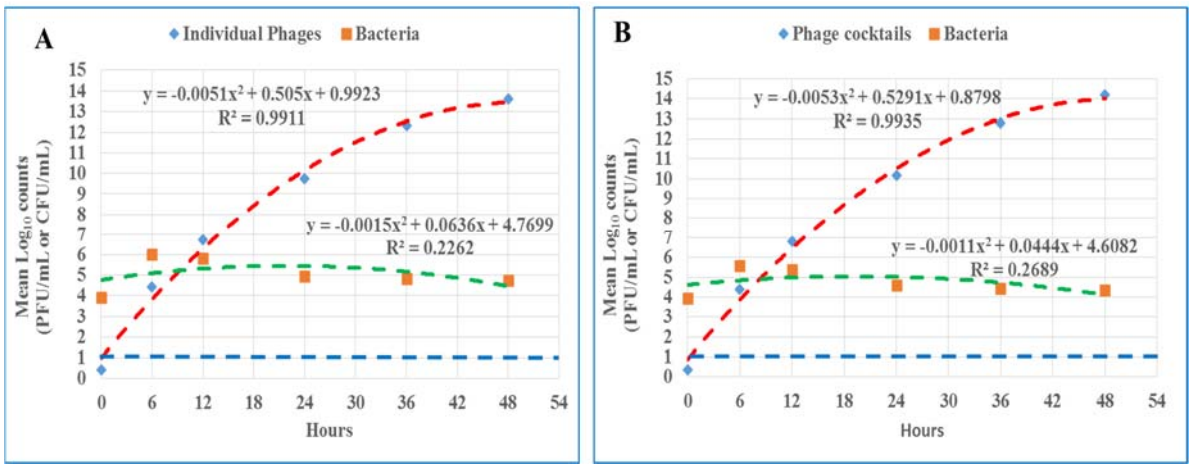

Figure 7.1: Mean $\log_{10}$ counts for individual phages, phage cocktails and bacteria cells incubated at 39 °C over 48 hours. **A** = Individual phages and the bacteria; **B** = phage cocktails and the bacteria. Dotted red and green lines represent phages titer ($\log_{10}$ PFU/mL) (Individual phage and cocktail) and bacteria concentration ($\log_{10}$ CFU/mL), respectively). Dotted blue lines represent detection limit for direct plating.

There was a significant quadratic response for both individual phages and their cocktails' titres at 39 °C over the 48-hour incubation period. Individual phage titres peaked at 50 to 52 hours of incubation as determined from prediction equations with R^2 values ranging between 0.811 to 0.994 (Table 7.2) while the titre of phage cocktails peaked between 51 and 55 hours of incubation as determined from prediction equations with R^2 values ranging from 0.982 to 0.995, Table 7.3. Phage vB_EcoM_3A1 titre peaked at 49 hours while phages vB_EcoM_10C2, vB_EcoM_118B, vB_EcoM_11B2, vB_EcoM_366B and vB_EcoM_366V titres were at a maximum at 50 hours of incubation. The titres for vB_EcoM_12A1 and vB_EcoM_10C3 reached a maximum at 52 and 53 hours, respectively. For phage cocktails, T1's titre peaked at 51 hours while the titres for T3 and T6 reached a maximum at 53 hours. The titre values for T5, T2 and T4 cocktails peaked at 52, 54 and 55 hours, respectively.

Table 7.2: Relationship between time (*x*) and individual phage titre (Log_{10} PFU, *y*) when incubated for 48 hours.

Phages	Regression equation	R^2	P-value	Time to peak titre (hrs)
vB_EcoM_10C2	$y = 1.128 (\pm 0.2139) + 0.501 (\pm 0.0237)x - 0.005 (\pm 0.0005)x^2$	0.985	<0.001	50
vB_EcoM_10C3	$y = 0.752 (\pm 0.1727) + 0.525 (\pm 0.0191)x - 0.005 (\pm 0.0004)x^2$	0.991	<0.001	53
vB_EcoM_118B	$y = 1.149 (\pm 0.1815) + 0.496 (\pm 0.0201)x - 0.005 (\pm 0.0004)x^2$	0.989	<0.001	50
vB_EcoM_11B2	$y = 1.036 (\pm 0.1403) + 0.502 (\pm 0.0155)x - 0.005 (\pm 0.0003)x^2$	0.994	<0.001	50
vB_EcoM_12A1	$y = 0.730 (\pm 0.1725) + 0.521 (\pm 0.0191)x - 0.005 (\pm 0.0004)x^2$	0.991	<0.001	52
vB_EcoM_366B	$y = 1.014 (\pm 0.2037) + 0.503 (\pm 0.0226)x - 0.005 (\pm 0.0005)x^2$	0.987	<0.001	50
vB_EcoM_366V	$y = 1.088 (\pm 0.2153) + 0.503 (\pm 0.0238)x - 0.005 (\pm 0.0005)x^2$	0.985	<0.001	50
vB_EcoM_3A1	$y = 1.041 (\pm 0.2006) + 0.489 (\pm 0.0222)x - 0.005 (\pm 0.0004)x^2$	0.811	<0.001	49

Table 7.3: Relationship between time (*x*) and phage cocktail titre (Log_{10} PFU, *y*) when incubated for 48 hours.

Phages	Regression equation	R^2	P-value	Time to peak titre (hrs)
T1	$y = 0.981 (\pm 0.1845) + 0.514 (\pm 0.0204)x - 0.005 (\pm 0.0004)x^2$	0.990	<0.001	51
T2	$y = 0.682 (\pm 0.1525) + 0.535 (\pm 0.0169)x - 0.005 (\pm 0.0003)x^2$	0.993	<0.001	54
T3	$y = 0.919 (\pm 0.1368) + 0.532 (\pm 0.0151)x - 0.005 (\pm 0.0003)x^2$	0.995	<0.001	53
T4	$y = 0.531 (\pm 0.1548) + 0.548 (\pm 0.0171)x - 0.005 (\pm 0.0003)x^2$	0.994	<0.001	55
T5	$y = 1.020 (\pm 0.2435) + 0.519 (\pm 0.0270)x - 0.005 (\pm 0.0005)x^2$	0.982	<0.001	52
T6	$y = 1.145 (\pm 0.2101) + 0.527 (\pm 0.0233)x - 0.005 (\pm 0.0005)x^2$	0.987	<0.001	53

7.3.2. Potency of phages against *E. coli* O177 cells in a simulated rumen environment

The effectiveness of phages in reducing *E. coli* O177 cell counts under rumen-simulated conditions was determined over a 48-hour incubation period. Response surface regression analysis revealed significant ($P < 0.05$) increase in percent reduction of *E. coli* O177 cells after exposure to individual phages and phage cocktails, Table 7.4-5. For individual phages, percent reduction of *E. coli* O177 cell counts peaked (60.81 - 63.27%) at 47 to 48 hours of incubation as determined from prediction equations with R^2 values ranging from 0.992 to 0.996. For phage cocktails, reduction of *E. coli* O177 cell counts peaked (63.06 - 73.25%) at 43 to 46 hours of incubation as determined from prediction equations with R^2 values ranging from 0.970 to 0.993. The effectiveness of individual phages, vB_EcoM_10C2, vB_EcoM_118B and vB_EcoM_12A1 against *E. coli* O177 cell counts peaked (61.031, 63.02 and 62.73%, respectively) at 48 hours of incubation while the other remaining phages peaked at 47 hours. For phage cocktails, T3 and T6, reduction of *E. coli* O177 cell counts (73.25 and 66.90%, respectively) reached a maximum at 43 hours whereas for T2 and T4, maximum reduction (63.80 and 67.18%, respectively) was achieved at 44 hours. For phage cocktail T1, relative reduction percentage of *E. coli* O177 cell counts peaked at 46 hours (66.06%) and for T5, relative reduction percentage of *E. coli* O177 cell counts maximised at 45 hours (63.06%).

Table 7.4: Relationship between time (*x*) and percent reduction of *E. coli* O177 cells (Log_{10} CFU, *y*) when exposed to individual phages.

Phages	Regression equation	R^2	P-value	Time to maximum reduction (hrs)
vB_EcoM_10C2	$y = 1.212 (\pm 0.6744) + 2.548 (\pm 0.0746)x - 0.027 (\pm 0.0015)x^2$	0.993	<0.001	48
vB_EcoM_10C3	$y = 1.258 (\pm 0.650) + 2.583 (\pm 0.0720)x - 0.028 (\pm 0.0014)x^2$	0.994	<0.001	47
vB_EcoM_118B	$y = 0.476 (\pm 0.7273) + 2.599 (\pm 0.0805)x - 0.027 (\pm 0.0016)x^2$	0.993	<0.001	48
vB_EcoM_11B2	$y = 1.054 (\pm 0.6771) + 2.646 (\pm 0.0749)x - 0.029 (\pm 0.0015)x^2$	0.993	<0.001	47
vB_EcoM_12A1	$y = 0.715 (\pm 0.677) + 2.636 (\pm 0.0749)x - 0.028 (\pm 0.0015)x^2$	0.993	<0.001	48
vB_EcoM_366B	$y = 1.930 (\pm 0.7607) + 2.621 (\pm 0.084)x - 0.028 (\pm 0.0017)x^2$	0.992	<0.001	47
vB_EcoM_366V	$y = 1.317 (\pm 0.6620) + 2.616 (\pm 0.0732)x - 0.028 (\pm 0.0014)x^2$	0.994	<0.001	47
vB_EcoM_3A1	$y = 0.680 (\pm 0.5571) + 2.636 (\pm 0.0616)x - 0.028 (\pm 0.0012)x^2$	0.996	<0.001	47

Table 7.5: Relationship between time (*x*) and percent reduction of *E. coli* O177 cells (Log_{10} CFU, *y*) when exposed to phage cocktails.

Phages	Regression equation	R^2	P-value	Time to maximum reduction (hrs)
T1	$y = 3.359\ (\pm 0.938) + 2.697\ (\pm 0.1038)x - 0.029\ (\pm 0.0021)x^2$	0.988	<0.001	46
T2	$y = 1.887\ (\pm 0.734) + 2.815\ (\pm 0.0813)x - 0.032\ (\pm 0.0016)x^2$	0.993	<0.001	44
T3	$y = 3.803\ (\pm 1.2326) + 2.905\ (\pm 0.1364)x - 0.034\ (\pm 0.0027)x^2$	0.981	<0.001	43
T4	$y = 5.049\ (\pm 1.510) + 2.864\ (\pm 0.167)x - 0.033\ (\pm 0.0033)x^2$	0.970	<0.001	44
T5	$y = 2.671\ (\pm 0.899) + 2.692\ (\pm 0.0995)x - 0.030\ (\pm 0.0020)x^2$	0.988	<0.001	45
T6	$y = 3.606\ (\pm 1.1709) + 2.977\ (\pm 0.1296)x - 0.035\ (\pm 0.0026)x^2$	0.983	<0.001	43

Both individual phages and phage cocktails significantly reduced *E. coli* O177 cell count over a 48-hour incubation period with their effectiveness ranging between 16.03 and 62.74% (from 6 to 48 hours) , Table 7.6-7. At 6 and 12 hours of incubation, the highest ($P < 0.05$) percent reduction in *E. coli* cell count was observed in samples treated with phage vB_EcoM_366B (19.41%) and vB_EcoM_10C3 (28.59%), respectively. At 12 hours, highest reduction percentage was seen in samples treated with vB_EcoM_10C3 and vB_EcoM_366B (28.59 and 28.61%, respectively), which did not differ. At 24 hours, phage vB_EcoM_11B2 was the most effective ($P < 0.05$) resulting in 50.10% reduction in *E. coli* while at 36 and 48 hours, the largest reduction (56.42 and 62.74%, respectively) was recoded in samples treated with the phage vB_EcoM_3A1 ($P < 0.05$). The effectiveness of phage cocktails in reducing *E. coli* counts ranged between 20.65 and 66.92% throughout the 48-hour incubation period. Phage T4 was the most effective at reducing *E. coli* counts at 6 (26.63%) and 12 hours (36.75%) while at 24 and 36 hours, the most effective phage cocktail (55.19 and 60.69%, respectively) was T6. At 48 hours, the most effective cocktail was T4 (66.92%). However, there was no significant difference in the effectiveness of cocktails T3, T4 and T6 (66.67, 66.92 and 62.74%, respectively) against *E. coli* cells.

Table 7.6: Percent reduction of *E. coli* O177 strain in a rumen model when exposed to individual phages.

Incubation time (hrs)	Individual phages								
	vB_EcoM_ 10C2	**vB_EcoM_ 10C3**	**vB_EcoM_ 118B**	**vB_EcoM_ 11B2**	**vB_EcoM_ 12A1**	**vB_EcoM_ 366B**	**vB_EcoM_ 366V**	**vB_EcoM_ 3A1**	**SEM**
0	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.368
6	17.25^{c}	16.58^{abc}	16.31^{ab}	16.89^{bc}	16.03^{a}	19.41^{e}	18.09^{d}	16.46^{ab}	0.168
12	27.03^{b}	28.59^{d}	25.86^{a}	27.66^{bc}	28.05^{cd}	28.61^{d}	27.92^{cd}	27.54^{bc}	0.194
24	48.37^{a}	48.53^{b}	49.56^{f}	50.10^{g}	49.60^{f}	49.52^{e}	49.07^{c}	49.50^{d}	0.123
36	54.37^{a}	54.43^{a}	55.13^{ab}	55.62^{bc}	54.96^{ab}	56.10^{c}	$55.78b^{c}$	56.42^{c}	0.207
48	61.02^{a}	61.12^{a}	61.69^{ab}	61.73^{ab}	61.89^{b}	62.31^{bc}	61.90^{b}	62.74^{c}	0.180

***Key*:** ^{a,b,c,d} means within a row that do not share a common superscripts differ significantly ($P < 0.05$); SEM = standard error mean.

Table 7.7: Percent reduction of *E. coli* O177 strain in a rumen model when exposed to phage cocktails.

Incubation time (hours)	Phage cocktails						
	T1	**T2**	**T3**	**T4**	**T5**	**T6**	**SEM**
0	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.368
6	21.79^{b}	20.65^{a}	24.38^{c}	26.63^{d}	21.05^{a}	24.65^{c}	0.168
12	32.52^{b}	30.29^{a}	34.83^{c}	36.75^{d}	30.73^{a}	34.69^{c}	0.194
24	50.81^{b}	51.51^{c}	54.34^{e}	53.51^{d}	50.27^{a}	55.19^{f}	0.123
36	58.60^{b}	58.67^{b}	59.84^{c}	59.81^{c}	56.41^{a}	60.69^{d}	0.207
48	65.34^{b}	62.82^{a}	66.67^{c}	66.92^{c}	62.35^{a}	66.42^{c}	0.180

***Key*:** ^{a,b,c,d,e,f} means within a row that do not share a common superscripts differ significantly ($P < 0.05$); SEM = standard error mean.

7.4. Discussion

Ruminants are considered to be principal reservoirs of the atypical enteropathogenic *E. coli* O177 strain (Montso *et al.*, 2019). The gastrointestinal tract (GIT) and hides of live ruminants, particularly cattle, carry significant numbers of foodborne pathogens such as *E. coli* O177 strain. In addition, *E. coli* species are predominantly found in the lower GIT of ruminants (Grauke *et al.*, 2002). However, there are factors such as pre-slaughter starvation, feeding high grain ration and subacute rumen acidosis that precipitate proliferation of *E. coli* in the rumen (Rasmussen *et al.*, 1993; Tkalcic *et al.*, 2000; Khafipour *et al.*, 2011). Consequently, this may increase populations of *E. coli* pathogens and thus exacerbate food contamination, especially during milking and slaughtering (Wang *et al.*, 2017). Against this background, there have been many attempts to evaluate the ability of phages (individual phages and/or cocktail) to reduce foodborne pathogen in live animals (Sheng *et al.*, 2006; Rozema *et al.*, 2009; Rivas *et al.*, 2010;

Raya *et al.*, 2011). Although *in vitro* studies have demonstrated significant effect of individual phages and/or cocktails in reducing *E. coli* cell counts, *in vivo* investigations have revealed mixed results (Sheng *et al.*, 2006; Rozema *et al.*, 2009). Application of phages via anal route in cattle did not reduce *E. coli* population and this could be due to the inability of phages to interact with the host when delivered this way (Rivas *et al.*, 2010; Cooper, 2016). However, *in vitro* rumen system model studies revealed significant reduction of *E. coli* O157 (Bach *et al.*, 2003; Rivas *et al.*, 2010) but similar studies have not been done for *E. coli* O177.

In the present study, the effectiveness of individual phages and phage cocktails in reducing *E. coli* O177 was evaluated in an *in vitro* ruminal fermentation system over a 48-hour incubation period. Quadratic trends in *E. coli* O177 cell counts in response to incubation time were observed for both individual phages and their cocktails. Phage forming units tended to increase with time while bacterial population tended to decrease. Bacterial load fluctuation presumably reflects the activity of phages in inhibiting *E. coli* O177 growth in the simulated rumen fermentation system. Phage vB_EcoM_3A1 needed the shortest incubation time (49 hours) to attain its peak titre while phage vB_EcoM_10C3 required the longest time (53 hour). This demonstrates that the phage vB_EcoM_3A1 performs better under rumen fermentation conditions. Therefore, for biocontrol purposes, vB_EcoM_3A1 could be a suitable candidate for developing phage therapy intended to control foodborne pathogens in live animals (Goodridge and Bisha, 2011).

On the other hand, phage cocktails achieved their maximum titres at predicted incubation times of 51 and 55 hours as determined from regression equation with R^2 values ranging from 0.982 to 0.995. Phage cocktail T1 required the shortest time to reach peak titre while phage cocktail T4 required the longest time. This suggests that T1 may have produced better results in

reducing *E. coli* O177 cells in the rumen fermentation system. Although phage cocktails required a longer period to reach their peak titres compared to individual phages, both phage types were stable under the rumen conditions (39 °C, pH 6.5, anaerobic condition with the presence of other rumen microbes and enzymes). This indicates that both phage types may be suitable for reducing *E. coli* O177 cells in the ruminants prior to slaughter (Goodridge and Bisha, 2011).

Percentage reduction in *E. coli* O177 cell counts peaked (60.81 to 63.27%) at 47 to 48 hours when treated with individual phages. On the other hand, reduction in *E. coli* O177 cell counts was maximized (63.06 to 73.25%) within a shorter period (43 to 46 hours) when exposed to phage cocktails. It is noteworthy that phage cocktails were not only more effective but also required less time compared to individual phages. This could be attributed to the fact that phage cocktails have wide-host range than individual phages (Cooper, 2016). Furthermore, phage resistance is likely to affect the efficacy of individual phages rather than phage cocktails (Labrie *et al.*, 2010).

Comparing individual phages, phage vB_EcoM_3A1 required the shortest time to reach its peak titre while showing the highest (62.74%) overall percentage reduction of *E. coli* cells after 48 hours of incubation. However, there was no significant difference between the effectiveness of vB_EcoM_366B and vB_EcoM_3A1 in reducing *E. coli* cells. In addition, other individual phages significantly reduced *E. coli* O177 cell counts. This demonstrated that phages were actively infecting the targeted bacteria. Although individual phages revealed significant reduction of *E. coli* O177 cell counts, the reduction was lower compared to those reported in previous studies (Bach *et al.*, 2003; Rivas *et al.*, 2010). This could be attributed to the fact the MOI (10) used in this study was 10 times lower than that of the previous the study by Rivas

and co-workers in 2010. In addition, the different fermentation substrates and targeted bacteria (*E. coli* O157) could perhaps account for the differences.

Phage cocktails T1 and T5 were the least effective against *E. coli* O177 while phage cocktails T3, T4 and T6 were the most effective over 48 hours of incubation. This suggests that T3, T4 and T6 are more suitable for the biocontrol of *E. coli* O177 in live animals. Interestingly, the cocktails were more effective in reducing bacterial load compared to individual phages. This could be attributed to the fact that phage cocktails target multiple host cell's receptors and thus infect *E. coli* O177, which might be resistant to individual phages (Labrie *et al.*, 2010; Goodridge and Bisha, 2011). Therefore, this characteristic showed that the phage cocktails are suitable for biocontrol of *E. coli* O177 in cattle.

7.5. Conclusion

Individual phages and their cocktails were stable and viable under rumen fermentation conditions. In addition, both individual phages and phage cocktails significantly reduced *E. coli* O177 cell counts in the rumen model system. However, phage cocktails were more effective compared to individual phages. This suggests that phage cocktails could be administered per os in live cattle to reduce *E. coli* O177 in the rumen, especially just before slaughter.

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CHAPTER EIGHT

WHOLE GENOME SEQUENCING OF A NOVEL LYTIC *ESCHERICHIA* PHAGE VB_ECOM_11B2-MVA

CHAPTER EIGHT

WHOLE GENOME SEQUENCING OF A NOVEL LYTIC *ESCHERICHIA* PHAGE VB_ECOM_11B2-MVA

Abstract

Lytic bacteriophages are considered as a viable alternative to antimicrobial agents against multi-drug resistant pathogens. Despite this, complete genome sequence is a prerequisite for lytic phage candidates intended for biocontrol application to confirm the absence of undesirable traits. Therefore, the objective of this study was to perform complete genome sequence of lytic vB_EcoM_11B2-MVA phage using PacBio RSII platform. Furthermore, to classify and genetically characterise *Escherichia* phage vB_EcoM_11B2-MVA genome.

The results revealed that *Escherichia* phage vB_EcoM_11B2-MVA genome was 152,234 bp linear dsDNA with 39.1% GC content. Furthermore, *Escherichia* phage vB_EcoM_11B2-MVA genome annotation revealed no lysogenic (integrase), virulence and antimicrobial resistance sequences. Furthermore, phage genome contained 30 genes encoding for phage proteins and 11 tRNA gene sequences coding for 10 amino acids. *Escherichia* phage vB_EcoM_11B2-MVA genome revealed high identity similarities with *E. coli* phages belonging to the order Caudovirale and *Myoviridae* family. In addition, phylogenomic and VICTOR analysis grouped *Escherichia* phage vB_EcoM_11B2-MVA genome with *Escherichia* phage vB_EcoM_Schickermooser. Based on these similarities, *Escherichia* phage vB_EcoM_11B2-MVA genome was classified under the order Caudovirales, *Myoviridae* family and the new genus "*Phapecoctavirus*". In conclusion, complete genome sequence analysis revealed that phage vB_EcoM_11B2 is safe and suitable to be used as biocontrol agent to reduce *E. coli* O177 strain both in live animals and food.

Keywords: Phage genome, *E. coli* O177, bacteriophage, biocontrol, antibiotic resistance

8.1. Introduction

Bacteriophages (known as phages) are viruses that infect and replicate within bacterial cells using the host's replication machinery (Harada *et al.*, 2018). They are the most abundant biological entities with an estimated number of 4.8 x 10^{31} phage particles on earth (Cobián Güemes *et al.*, 2016; Stone *et al.*, 2019). Furthermore, phages are diverse in their biological, structural and physicochemical properties (Ackermann, 2007; Ye *et al.*, 2019). Phage structure comprises of a three-dimensional form, which is made up of an icosahedral protein capsid either with or without a tail and a filament (Ackermann, 2007; Rao and Lalitha, 2015; Ye *et al.*, 2019).

The International Committee on Taxonomy of Virus (ICTV) body is responsible for classification of bacteriophages (Sharma *et al.*, 2017). The ICTV classify phages rely on host, transmission electron microscopy (TEM) morphotype and type of nucleic acid (Ackermann, 2007; Low *et al.*, 2019). However, whole genome sequencing (WGS) is regarded as a "gold standard" for classification of phages (Jagadeesan *et al.*, 2019). Based on these, there are 22 bacterial and archaeal bacteriophages families (https://talk.ictvonline.org/taxonomy/) (Stone *et al.*, 2019) and the majority (13) of these phage families belonged to the order Caudovirales (Ackermann, 2007). Despite the fact that more than 96% of phages are tailed phages that contain dsDNA, they vary with respect to their shapes, filamentous and pleomorphic forms (Kakasis and Panitsa, 2019). Phages belonging to the families *Myoviridae* (long contractile tail), *Siphoviridae* (long non-contractile tail) and *Podoviridae* (short non-contractile tail) are the most common dsDNA phages found in the environment (Harada *et al.*, 2018; Stone *et al.*, 2019).

Although bacteriophages are to be the most ubiquitous (4.8×10^{31}) entities on earth with ten times more bacteria population (Cobián Güemes *et al.*, 2016; Stone *et al.*, 2019), only a small fraction of phage genomes are well characterised and taxonomically classified (Low *et al.*, 2019). Based on WGS analysis, 85 genera with 2047 phage species under *Myoviridae* have been reported in the NCBI database [https://www.ncbi.nlm.nih.gov/Taxonomy (accessed on 04, November 2019)]. A small (10.8%) proportion of phage species (genomes) belongs to *Escherichia* phages [https://www.ncbi.nlm.nih.gov/Taxonomy (accessed on 04, November 2019)]. Despite this, 11.8% of the *Escherichia* phage genomes reported in the NCBI are unclassified [https://www.ncbi.nlm.nih.gov/Taxonomy (accessed on 04, November 2019)]. This is attributed to lack of phenotype information (Low *et al.*, 2019). Against this background, new methods such as Virus Classification and Tree Building Online Resource (VICTOR) have combined with WGS to classify new phages (https://ggdc.dsmz.de/victor.php) (Meier-Kolthoff and Göker, 2017).

Given that lytic phages are considered as viable alternative to improve food safety and controlling antimicrobial resistance pathogens, there is a need to expand our knowledge on lytic phages (Korf *et al.*, 2019). Whole genome sequencing is a prerequisite to confirm the safety properties of phage candidates for therapeutic purposes (Joensen *et al.*, 2014; Yokoyama *et al.*, 2018; Bai *et al.*, 2019; Yang *et al.*, 2019). Furthermore, WGS is ideal for providing not just an overview of the genome map but essential to screen for the absence of undesirables gene segments within the phage genome (Jagadeesan *et al.*, 2019). Hence, the purpose of this study was to determine genetic characteristics of the phage vB_EcoM_11B2 genome as a novel agent for the *E. coli* O177 strain.

8.2. Materials and methods

8.2.1. Preparation of *Escherichia coli* O177 culture

Antibiotic resistant atypical enteropathogenic *E. coli* O177 strain (isolated in this study) was used in the *in vitro* ruminal fermentation system. Briefly, a pure culture of *E. coli* O177 was removed from −80 °C freezer and revived on MacConkey agar. The plates were incubated at 37 °C for 24 hours. After incubation, a single colony was transferred into 50 mL sterile falcon tube containing 10 mL nutrient broth and the tube was also incubated at 37 °C for 24 hours. After incubation, the optical density (OD at 630mn) was measured using spectrophotometer. Nutrient broth was used to adjust the OD to 0.5 McFarland standard.

8.2.2. Propagation phage vB_EcoM_11B2 for DNA extraction

The vB_EcoM_11B2 phage isolated in this study was propagated using *E. coli* O177 strain for phage DNA extraction. Phage propagation was performed as previously described (Sambrook and Russell, 2001). In brief, 100 µL (0.5 McFarland standard) of overnight culture of *E. coli* O177 was added into a 50 mL falcon tube containing 30 mL tryptic soya broth supplemented with 10 mM magnesium sulphate (MgSO4 7H_2O). Aliquot of 100 µL of each phage was added to tube. The tube was incubated at 37 °C for 24 hours in a shaking incubator (80 rpm). After incubation, the tube was centrifuged at 10 000 × g for 10 minutes at 4 °C. The supernatant was filter-sterilised using 0.22 µm pore-size acrodisc syringe filter. Phage titer was determined using plaque assay.

8.2.4. Phage DNA extraction

Phage DNA was extracted from vB_EcoM_11B2 stock (3 x 10^{11} PFU/mL using phenol-chloroform-isoamyl alcohol method as previously described (Nale *et al.*, 2016), with the modifications. Briefly, phage stock was precipitated with 10% (w/v) polyethylene glycol

(PEG) 8000 and the mixture was incubated at 4 °C overnight to allow precipitation of phage particles. Precipitated phage was centrifuged at 10000 × g for 10 minutes at 4 °C to sediment phage particles and the supernatant was discarded. The phage pellet was washed three using lambda diluent [5.8 g/L NaCl, 2 g/L MgSO4.7H_2O, 10ml/L Tris-HCl (pH 7.5)] to remove residual PEG. The pellet was re-suspended in 500 µL of lambda diluent. The sample was treated with DNase I (0.8U/mL) (Organic, NJ, USA) and RNase A (0.1 mg/mL (Sigma Aldrich, USA) (final concentration) for 30 minutes at 37 °C to degrade and eliminate bacterial DNA and RNA.

After incubation, 20% (w/v) sodium dodecyl sulfate (SDS) (Fisher Scientific, UK) and 10 mg/mL proteinase K (Sigma Aldrich, USA) were added to the sample. The mixture was inverted three times and incubated for 30 minutes at 37 °C. After incubation, aliquots of 500 µL were transferred into 4 x 1.5 µL eppendorf tubes. Aliquots of 500 µL phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) equal volumes were added to the tubes. The tubes were inverted five times and centrifuged at 10000 × g for 5 minutes at 4 °C. The aqueous layer was removed into a new 1.5 µL eppendorf tubes and phenol-chloroform-isoamyl alcohol step was repeated. The aqueous layer was transferred to a new 1.5 µL eppendorf tubes and 500 µL chloroform and isoamyl (24:1, v/v) equal volumes were added to the tubes. The mixture was centrifuged at 10000 × g for 5 minutes at 4 °C. The aqueous layer was transferred to a new eppendorf tube and phage DNA was precipitated by adding 45 µL of 3 M sodium acetate (pH 5.2) and 500 µL absolute isopropanol. The mixture was incubated overnight at -20 °C to precipitate phage DNA. After incubation, the mixture was centrifuged at 14800 x g for 20 minutes. The pellet was washed twice with 500 µL chilled 70% (v/v) ethanol. The pellet was resuspended in 30 µL TE (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) buffer.

8.2.5. Phage DNA purification and quantity

Phage genomic DNA was purified using Norgen phage DNA isolation kit (Norgen Bioteck Corp., Ontario Canada), following the manufacturer's instructions. DNA quality was assessed using Nanodrop^{TM} -Lite Spectrophotometer (Thermo Fisher Scientific Limited).

8.2.6. Whole genome sequence of phage vB_EcoM_11B2

8.2.6.1. Library preparation and sequencing

Phage DNA was sequenced at Inqaba Biotechnical Industry (Pty) (Pretoria, South Africa) in South Africa. Briefly, phage genomic DNA was sequenced on a PacBio RSII platform (Pacific Biosciences, USA) following the manufacturer's protocol. The Quibit fluorometer and NanoDrop were used to determine the quantity and quality of the phage DNA sample. The sample was fragmented using Covaris g-TUBE shearing device and then purified with AMPure beads (Beckman Coulter, USA). The fragmented DNA sample was used to construct the library using PacBio SMRTbell library (BluePippin System 0.75% DF Marker U1 high-pass 30-40 kb v3) kit following the manufacturer's standard protocol. A 6584× sequence depth coverage constituting 1.83 GB file with 2 x 300 bp long paired-end reads were generated. The quality of the raw reads were assessed by FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/) (Andrews, 2010). The quality reads were filtered and the barcodes used were trimmed off using Trimmomatic (with parameters set at default) and the sample was de-multiplexed (Bolger *et al.*, 2014). *De novo* assembly of the trimmed reads was carried out using Hierarchical Genome Assembly Process 4 (HGAP4) SMRTLINK v7.0.1 and 154 kb data was generated. The phage genome was assigned a name "*Escherichia* phage vB_EcoM_11B2-MVA" for further analyses and this name will be used henceforth.

8.2.6.2. Bioinformatics analysis and annotation of *Escherichia* phage vB_EcoM_11B2-MVA genome

The assembled genome of *Escherichia* phage vB_EcoM_11B2-MVA was annotated using the Rapid Annotation Using Subsystem Technology (RAST) (version 2.0) server (Aziz *et al.*, 2008), Pathosystems Resource Integration Center (PATRIC, version 3.5.43) (https://patricbrc.org/) and Phage Search Tool (PHAST) (Zhou *et al.*, 2011) online tools. The genomes were searched for virulence and antibiotic resistance genes using VirulenceFinder version 2.0 (Joensen *et al.*, 2014) and ResFinder version 2.2 (Zankari *et al.*, 2012), respectively (all at 95% ID threshold), which are online tools developed by the Center for Genomic Epidemiology (CGE) (www.genomicepidemiology.org). Open reading frames (ORFs) were predicted using ORF Finder (with all parameters set at default values and ATG as start codon) (www.ncbi.nlm.nih.gov/orffinder/) and annotated functions of putative ORFs were confirmed using BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A search for phage encoded tRNA genes was done using tRANscan-SE program (version 2.0) (lowelab.ucsc.edu/cgi-bin/tRNAscan-SE2.cgi) with parameters set at default values (Lowe and Chan, 2016).

The GC and GC skew content of *Escherichia* phage vB_EcoM_11B2-MVA genome was identified using CGView tool (http://stothard.afns.ualberta.ca/cgview_server) (Stothard and Wishart, 2004) and circular genome map was generated using Blast Ring Generator (BRIG) platform (https://sourceforge.net/projects/brig/). Genome annotation was curated by blast search analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Predicted proteins in the genome were further annotated using BLASTP against the NCBI non-redundant GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1997).

Escherichia phage vB_EcoM_11B2-MVA) genome was deposited into blast search platform (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine genome identity similarities with known phage genomes from NCBI database. The nucleotide sequences of seven phage genomes showing 97.53 to 99.16% identity similarities with 91 to 95% query cover to *Escherichia* phage vB_EcoM_11B2-MVA genome sequence were selected as references for further analysis Table 8.1. Phage sequence data (FSTA files) was retrieved from NCBI database. The sequence data (genomes) together with *Escherichia* phage vB_EcoM_11B2-MVA genome sequence were deposited into Virus Classification and Tree Building Online Resource (VICTOR) (https://ggdc.dsmz.de/victor.php) to determine taxonomic classification of newly isolated phage (*Escherichia* phage vB_EcoM_11B2-MVA) genome.

8.2.6.3. Phylogenetic analysis

The nucleotide sequence of *Escherichia* phage vB_EcoM_11B2-MVA genome was deposited into the NCBI database and the blast search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to determine genome similarities. The nucleotide sequence data of seven phage genomes (Table 8.1) with high identity similarities (97.53 to 99.16%) and query cover (91 to 95%) to *Escherichia* phage vB_EcoM_11B2-MVA genome sequence was used to infer phylogenetic position and type of *Escherichia* phage vB_EcoM_11B2-MVA. The phylogenetic tree based on the neighbour-joining and maximum likelihood algorithm was constructed using genetic analysis software MEGA 7 (Version 7.0.18) (Tamura *et al.*, 2013).

All pairwise comparisons of the nucleotide sequences *Escherichia* phage vB_EcoM_11B2-MVA genome and seven phage genome sequences listed in Table 8.1, were conducted using the Genome-BLAST Distance Phylogeny (GBDP) method as previously described (Meier-Kolthoff *et al.*, 2013) under the setting recommended for prokaryotic viruses (Meier-Kolthoff

and Göker, 2017). The resulting intergenomic distances were used to generate a balanced minimum evolution tree with branch support using FASTME and SPR post-processing for the formula D_4 (Lefort *et al.*, 2015). The *Escherichia* phage vB_EcoM_11B2-MVA genome with seven phage genome sequences (Table 8.1) were further subjected to JSpeicesWS (http://jspecies.ribohost.com/jspeciesws/) and pairwise comparisons were conducted to determine average nucleotide identity based on BLAST + (ANIb) calculation and tetra nucleotide correlation index as previously described (Richter *et al.*, 2015).

Table 8.1: List of phage genomes selected from NCBI database for phylogenetic analysis.

Phage genomes	**M.S.**	**T.S.**	**Q.C. (%)**	**E.V.**	**ID (%)**	**Accession**
***Escherichia* phage vB_EcoM-Ro121c4YLVW**	65690	2.511e+05	95	0.0	99.16	MH051333
***Escherichia* phage ESCO13**	64921	2.492e+05	94	0.0	99.03	KX552041
***Escherichia* phage vB_EcoM_Schickermooser**	64641	2.520e+05	95	0.0	99.02	MK373788
***Escherichia* phage vB_EcoM-Ro121lw**	64752	2.527e+05	95	0.0	98.94	MH160766
***Escherichia* phage ESCO5**	51735	2.338e+05	91	0.0	98.26	KX664695
***Escherichia* phage phAPEC8**	54767	2.348e+05	91	0.0	97.77	JX561091
***Klebsiella* phage ZCKP1**	53842	2.396e+05	92	0.0	97.53	MH252123

***Key*:** MS = Maximum score; TS = Total score; QC = Query cover, EV = E value; ID = Identity.

8.3. Results

8.3.1. Genomic features of *Escherichia* phage vB_EcoM_11B2-MVA

Whole genome sequence was performed using PacBio RSII platform to determine the characteristics of *Escherichia* phage vB_EcoM_11B2-MVA genome sequence, relatedness to known phages, taxonomy and specific features. *Escherichia* phage vB_EcoM_Schickermooser genome (GenBank accession number; MK373788) was used as reference. Table 8. 2 shows

genomic features of *Escherichia* vB_EcoM_11B2-MVA and *Escherichia* phage vB_EcoM_Schickermooser. *Escherichia* phage vB_EcoM_11B2-MVA genome was composed of linear double stranded DNA (dsDNA) molecule with 152,234 bp length and 39.1% GC content. *Escherichia* phage vB_EcoM_11B2-MVA genome contained 286 genes. A large proportion 275 (96.1%) of the genes were coding for proteins and 11 (3.8%) were coding for tRNA. A large proportion 233 (87.7%) of protein coding genes were hypothetical proteins while 42 (15.3%) were known proteins. *Escherichia* phage vB_EcoM_11B2-MVA genome contained 338 ORFs with 30 (8.9%) predicted to encode for functional proteins (putative proteins associated with phages), 11 (3.3%) encode for tRNA. A large proportion 233 (68.9%) of ORFs predicted to encode for hypothetical proteins while 52 (15.4%) ORFs produced no hit on BLASTp. Phage genome was annotated for the presence of transfer RNA (tRNA) secondary structures using tRNA-scan tool. *Escherichia* phage vB_EcoM_11B2-MVA genome contained 11 tRNA sequences (74-88 bp) coding for different amino acids, Table 8. 3.

Based on VirulenceFinder, ResFinder, RAST and Patric annotation, no toxin proteins, elements associated with lysogeny (integrase), virulence (such as *stx*) and antimicrobial genes or sequences were found in *Escherichia* phage vB_EcoM_11B2-MVA genome. As depicted in Figure 8.1A, *Escherichia* phage vB_EcoM_11B2-MVA genome contained 8 cell wall and capsule (conserved proteins), 3 phage features (phage tail fibre and phage capsid proteins) and 4 nucleotides and nucleosides. The reference phage (*Escherichia* phage vB_EcoM_Schickermooser) genome contained 8 cell wall and capsule, 1 phage features (phage tail fibre) and 4 nucleotides and nucleosides, Figure 8.1B. The entire *Escherichia* phage vB_EcoM_11B2-MVA genome map is depicted in Figure 8.2.

Table 8.2: General genome features of *Escherichia* phage vB_EcoM_11B2-MVA and *Escherichia* phage vB_EcoM_Schickermooser.

Features	***Escherichia* phage vB_EcoM_11B2-MVA**	***Escherichia* phage vB_EcoM_Schickermooser***
Order	Caudovirales	Caudovirales
Family	*Myoviridae*	*Myoviridae*
Taxonomic classification at genus level	unknown	unknown
Nucleic acid	Linear dsDNA	Linear dsDNA
Accession number	awaiting	MK373788
Genome size (bp)	152234	151194
Total number of Contig	1	1
G+C content (%)	39.1	39.0
Total number of genes	286	294
Total number of gene coding for proteins	275	284
Total number of ORFs	338	249
Total number tRNA	11	10

***Key*:** Asterik denotes phage used as reference for taxonomic classification of *Escherichia* phage vB_EcoM_11B2-MVA genome.

Table 8.3: Properties of tRNAs found in *Escherichia* phage vB_EcoM_11B2-MVA genome.

tRNA type	Strand	Length (bp)	tRNA Begin	tRNA End	Anticodon
Ile2	-	75	84036	83962	CAT
Arg	-	75	83959	83885	TCT
Ser	-	86	83554	83469	GCT
Tyr	-	88	83338	83251	GTA
Asn	-	86	83244	83159	GTT
Thr	-	75	83068	82994	TGT
Gly	-	74	82679	82606	TCC
Gln	-	76	82507	82432	TTG
Pro	-	77	82338	82262	TGG
Ile	-	74	82255	82182	GAT
Met	-	79	82103	82025	CAT

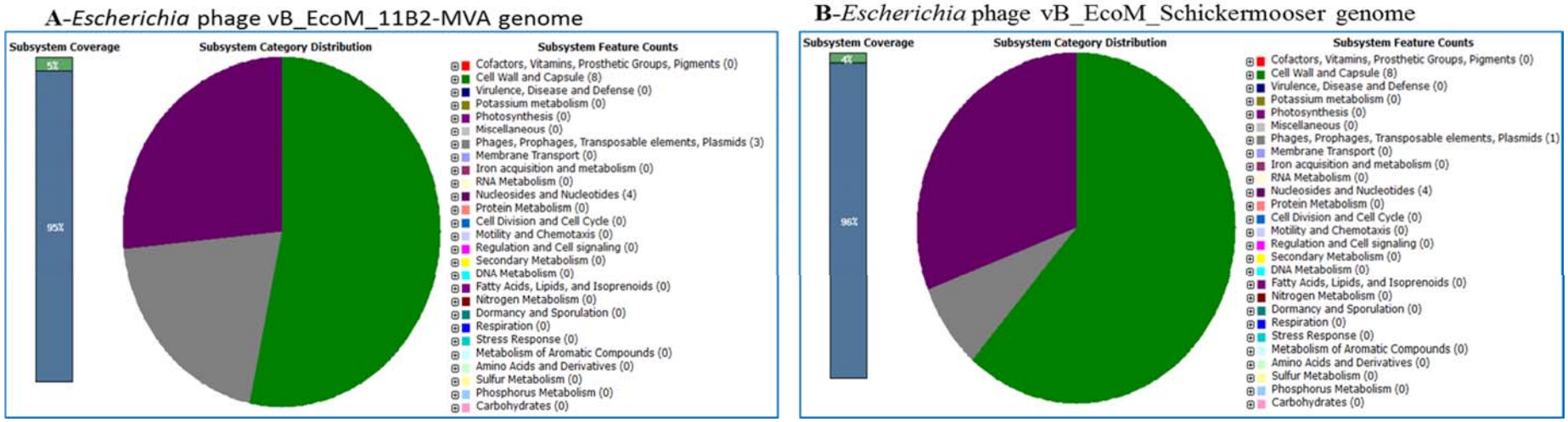

Figure 8.1: *Escherichia* phage genome features connect to subsystem and their distribution in different categories (*Escherichia* phage vB_EcoM_11B2-MVA and *Escherichia* phage vB_EcoM_Schickermooser genomes).

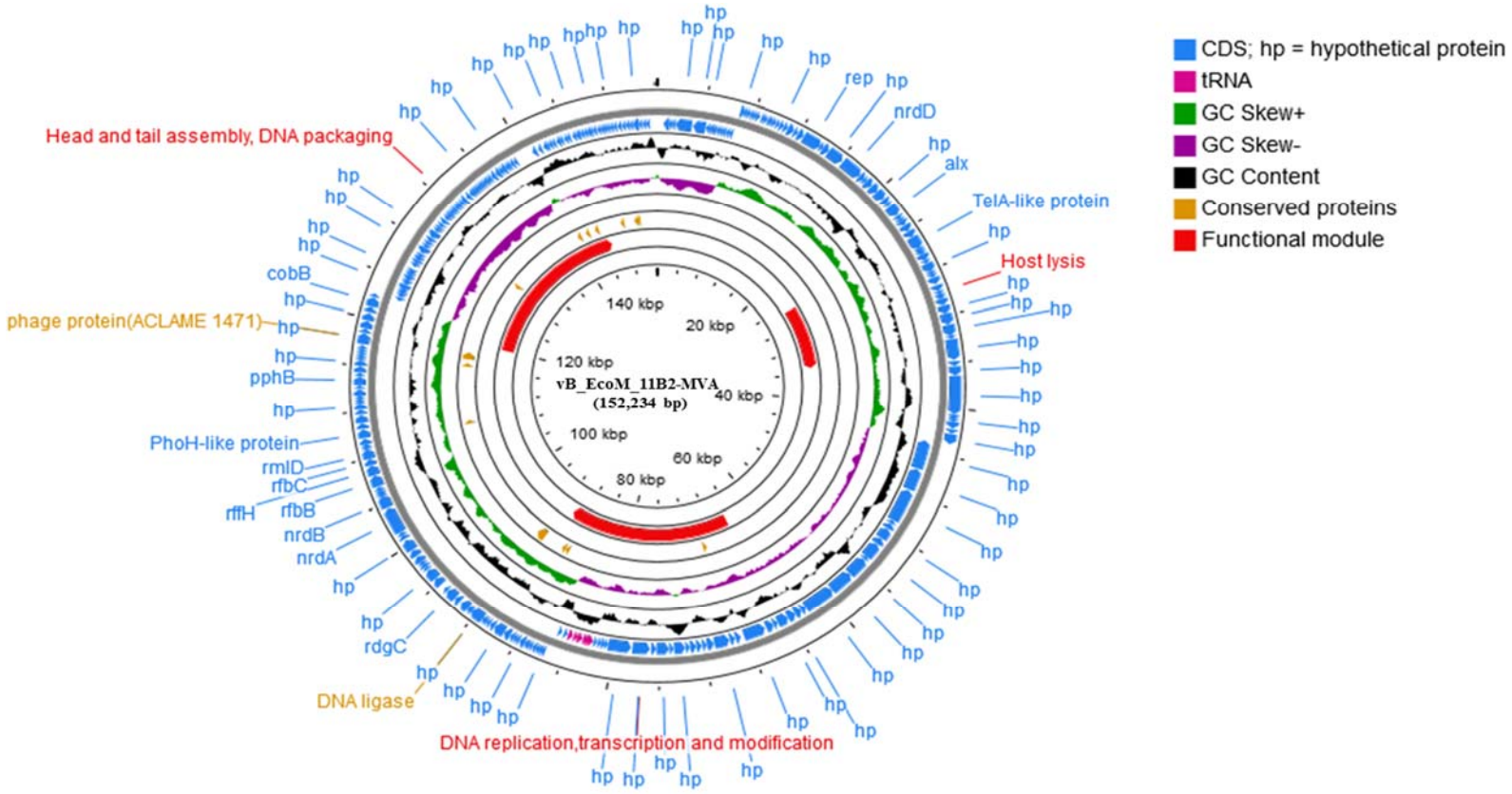

Figure 8.2: CGView (Circular Genome Viewer) image showing genomic map of *Escherichia* phage vB_EcoM_11B2-MVA. The coding regions are presented by arrows. The innermost rings show functional module (red arrows), conserved proteins (camel arrows), GC skew, (+green) and (– purple) and GC content (black). The two outermost rings depict tRNA (pink arrows) protein coding regions (blue arrows) of the genome.

8.3.2. Comparative genome analysis

BLASTn analysis of *Escherichia* vB_EcoM_11B2-MVA genome revealed 97.53-99.16% identity similarities to *Escherichia* phage vB_EcoM-Ro121c4YLVW, *Escherichia* phage ESCO13, *Escherichia* phage vB_EcoM-Ro121lw, *Escherichia* phage vB_EcoM_Schickermooser, *Escherichia* phage phAPEC8, *Escherichia* phage ESCO5 and *Klebsiella* phage ZCKP1 (accession numbers; MH051333, KX552041, MH160766, MK373788, JX561091, KX664695 and MH252123, respectively). Pairwise alignment was performed to assess *Escherichia* vB_EcoM_11B2-MVA genome similarities with *Escherichia* phage vB_EcoM_Schickermooser and *Escherichia* phage phAPEC8 genomes at nucleotide level. *Escherichia* vB_EcoM_11B2-MVA genome alignment sequence demonstrated 99.02% homology and 95% coverage with *Escherichia* phage vB_EcoM_Schickermooser genome sequence, Figure 8.3A. On the other hand, *Escherichia* vB_EcoM_11B2-MVA genome sequence alignment demonstrated 97.77% homology and 91% coverage with *Escherichia* phage phAPEC8 genome sequence, Figure 8.3B. The dotplot analysis indicated few (3) break points with short gaps in the diagonal line of *Escherichia* vB_EcoM_11B2-MVA vs *Escherichia* phage phAPEC8 genome sequences than in the diagonal line of *Escherichia* vB_EcoM_11B2-MVA vs *Escherichia* phage vB_EcoM_Schickermooser genome sequence. Comparative JSpeciesWS analysis revealed that *Escherichia* vB_EcoM_11B2-MVA genome and the above reference phage genomes shared 96.33-97.59% average nucleotide identity based on Blast (ANIb) (cut-off > 95%) with 0.996 TCS correlation index (cut-off > 0.999). Table 8. 4 shows genetic features of *Escherichia* phage vB_EcoM_11B2-MVA genomes and seven selected phage genome sequences analysed by JSpeciesWS tool. The genome size range from 147737 to 152234bp with 1 contig and 39.0 to 39.10% GC content.

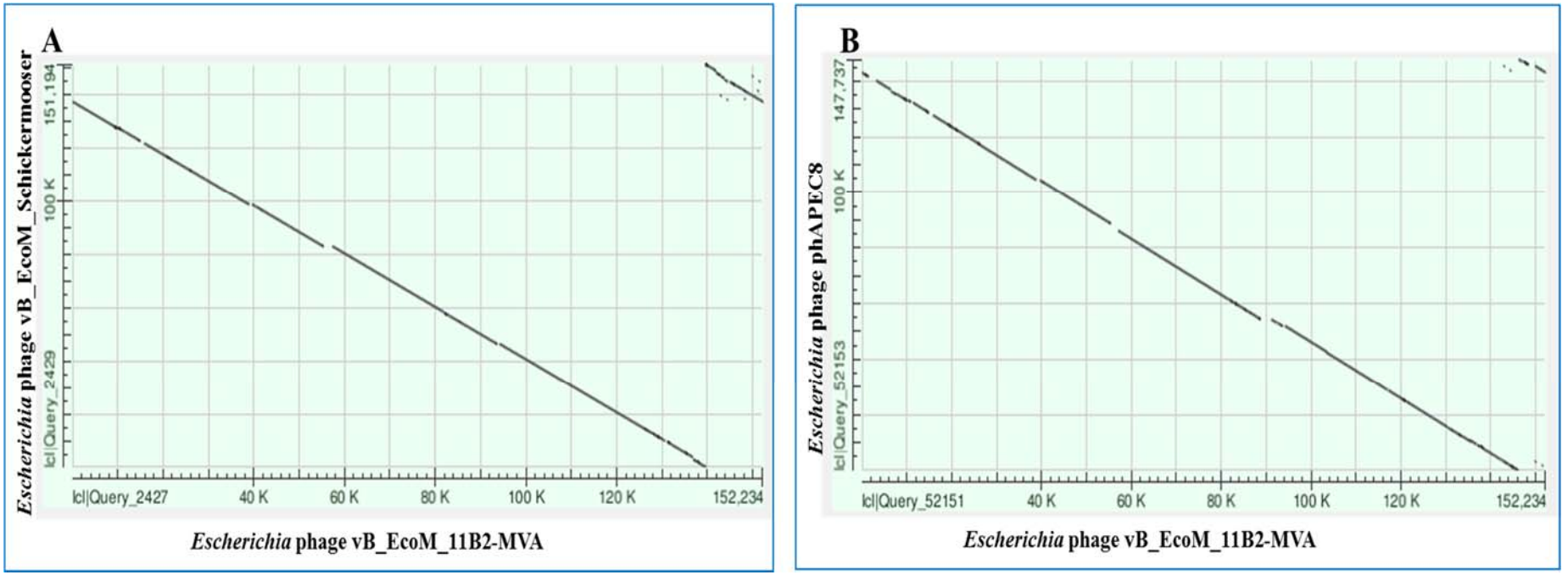

Figure 8.3: Dot matrix view of the BLASTn results showing regions of similarities of *Escherichia* phage vB_EcoM_11B2-MVA genome to other *Escherichia* phage genomes. *Escherichia* phage vB_EcoM_Schickermooser (**A**) and *Escherichia* phage vB_EcoM_11B2-MVA vs *Escherichia* phage phAPEC8 (**B**). The query sequence is represented on the X-axis and the numbers represent the bases/residues of the query. The subject is represented on the Y-axis and again the numbers represent the bases/residues of the subject. Alignments are shown in the plot as lines. The number of lines shown in the plot is the same as the number of alignments found by BLAST.

Table 8.4: Features of *Escherichia* phage vB_EcoM_11B2-MVA genome and other genetically related phage genomes.

Genome	Size (bp)	contigs	GC (%)
***Escherichia* phage vB_EcoM_11B2-MVA**	152234	1	39.10
***Escherichia* phage vB_EcoM-Ro121c4YLVW**	151671	1	39.10
***Escherichia* phage ESCO13**	149813	1	39.10
***Escherichia* phage vB_EcoM-Ro121lw**	149803	1	39.10
***Escherichia* phage vB_EcoM_Schickermooser**	151194	1	39.0
***Escherichia phage* _ESCO5**	149312	1	39.0
***Escherichia* phage phAPEC8**	147737	1	39.10
***Klebsiella* phage ZCKP1**	150925	1	39.10

8.3.3. Phylogenetic Analysis

Based on genomic comparative analysis using BLASTn algorithm, 7 phages showing 97.53-99.16% identity similarities with a query coverage of 91-95% and 0 E value were selected to determine their phylogenetic relationship with *Escherichia* phage vB_EcoM_11B2-MVA. As depicted in Figure 8.4A, phylogenetic analysis indicated that *Escherichia* phage vB_EcoM_11B2-MVA was in the same clade with six phages (5 *Escherichia* phages and 1 *Klebsiella* phage) belonging to *Myoviridae* family. *Escherichia* phage vB_EcoM_11B2-MVA clustered with *Escherichia* phage vB_EcoM_Schickermooser (GenBank accession number MK373788). The phylogenomic GBDP tree constructed using VICTOR tool, *Escherichia* phage vB_EcoM_11B2-MVA was placed with *Escherichia* phage vB_EcoM_Schickermooser, Figure 8.4B. *Klebsiella* phage ZCKP1 genome was not included in phylogenomic GBDP tree because of low score (value < 85%).

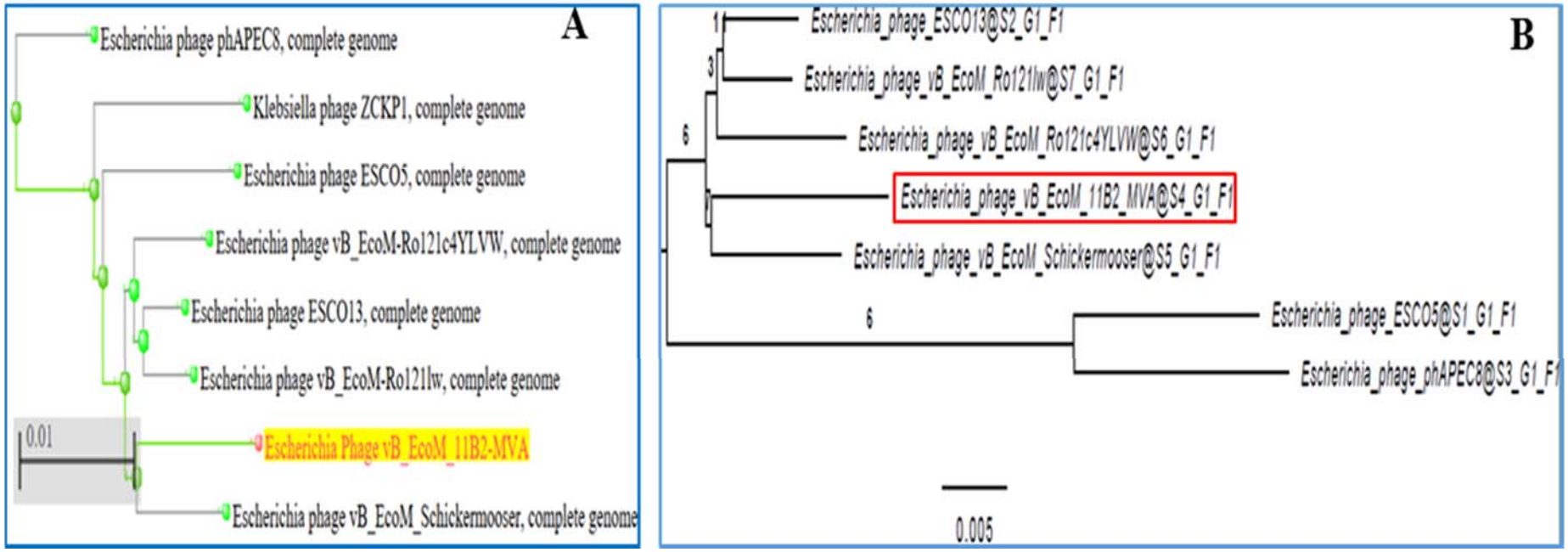

Figure 8.4: Phylogenetic tree of *Escherichia* phage vB_EcoM_11B2-MVA constructed based on the complete genome sequences of selected phages showing 95 to 99.6% identity similarity from NCBI-BLAST database (**A**). The tree was produced using Blast pairwise alignment using Neighbor-Joining method and the query sequence is highlighted in yellow. Phylogenomic GBDP tree of *Escherichia* phage vB_EcoM_11B2-MVA (circled in red) and *Myoviridae* members constructed by the VICTOR program (**B**). The recommended VICTOR tree (formula d_4) is shown and the numbers above the branches are GBDP pseudo-bootstrap support values from 100 replications, given that branch support exceeds 50%. The branch length of the resulting VICTOR tree is scaled in terms of the used distance formula.

8.4. Discussion

Bacteriophages have been considered as green technology for control of foodborne pathogens (Moye *et al.*, 2018). In the context of biocontrol application, selection of suitable lytic phage candidates for phage therapy is critical. Phages intended for biocontrol application, especially in food or live animals must have broad host range, high adsorption rate, short latent period and large burst size (Pereira *et al.*, 2016; Manohar *et al.*, 2019). In addition, precautionary measures must be enforced to avoid lysogenic or pseudo-lytic phages with virulence and antibiotic resistance genes. Although several *in vitro* tests have been employed to characterise phages based on physical parameters, whole genome sequence (WGS) is considered as "gold standard" to assess the safety of phages (Kwong *et al.*, 2015). In addition, whole genome sequence provides robust information such as absence or presence of lysogeny (integrase), virulence and antimicrobial resistance gene sequences in phage genomes (Rantsiou *et al.*, 2018; Jagadeesan *et al.*, 2019).

In this study, WGS was performed to determine genetic characteristics of the *Escherichia* phage vB_EcoM_11B2-MVA genome. The genome size was 152,234 bp with 39.10% GC content. Phage genome GC content was 22.7% lower than that of its host bacteria (*E. coli* O177) reported in this study (Chapter 4). In addition, the *Escherichia* phage vB_EcoM_11B2-MVA genome was linear dsDNA. The *Escherichia* phage vB_EcoM_11B2-MVA genome revealed high (97.53 to 99.16%) identity similarities with *Escherichia* phages, especially the *Escherichia* phage vB_EcoM_Schickermooser, which belongs to the *Myoviridae* family (Tsonos *et al.*, 2012; Trotereau *et al.*, 2017; Korf *et al.*, 2019). Moreover, the *Escherichia* phage vB_EcoM_11B2-MVA genome contained features similar (value > 99.7%) to the *Escherichia* phage vB_EcoM_Schickermooser. Based on these similarities, the *Escherichia* phage

vB_EcoM_11B2-MVA genome was classified under the order Caudovirales and *Myoviridae* family. This finding is consistent with TEM results obtained in this study (Chapter 4).

The *Escherichia* phage vB_EcoM_11B2-MVA genome contained 286 genes and 275 encoded proteins while 11 encoded tRNA. Furthermore, 233 were encoding hypothetical proteins, while 42 were coding for known proteins and 30 genes encoded functional proteins associated with phages. These findings are similarly to most of the previous phage studies, which reported more than 50% hypothetical proteins in lytic phage genomes (Altamirano and Barr, 2019; Korf *et al.*, 2019; Manohar *et al.*, 2019). *Escherichia* phage vB_EcoM_11B2-MVA genome annotation using ORFFinder predicted 338 ORFs. Among the 338 ORFs obtained, 30 were functionally annotated to encode phage proteins. These phage proteins were categorised into four functional modules [DNA replication/transcription modification (DNA ligase, DNA primase/helicase, DNA polymerase I, DNA mismatch repair endonuclease), structural and packaging proteins (capsid proteins, putative phage tail fibre protein, tail spike protein and terminase small and large subunits), host lysis (lysozyme) and other functional (Glucose-1-phosphate thymidylyltransferase)]. Majority (69.2%) of the annotated ORFs were encoding hypothetical proteins with unknown functions. These findings are consistent with the previous studies (Tsonos *et al.*, 2012; Korf *et al.*, 2019). In addition, ORFs predicted for hypothetical proteins, revealed high (> 95%) identity similarities with all *Escherichia* phage genome references used in this study. However, 15.4% of the annotated ORFs revealed no hit for protein coding on BLASTp.

Although some studies reported few or no tRNA genes in phage genomes (El-Dougdoug *et al.*, 2019; Yuan *et al.*, 2019), *Escherichia* phage vB_EcoM_11B2-MVA genome annotation using tRNA-scan revealed 11 tRNA sequences were coding for 10 different amino acids. In contrary

to the reference genome, *Escherichia* phage vB_EcoM_Schickermooser, this newly isolated genome had an extra copy of tRNA-Ile amino acid. Similar findings were observed in *Escherichia* phage vB_EcoM_Schickermooser and *Escherichia* phage phAPEC8 genomes (Tsonos *et al.*, 2012; Korf *et al.*, 2019). Despite the fact that phages utilise host cell's machinery for replication, presence of tRNAs in phage genome suggests that they complement host cell with their own genetic information to attain fitness and bias for synthesis of phage proteins (Bailly-Bechet *et al.*, 2007; Liao *et al.*, 2019). Furthermore, phages containing more tRNAs in their genome are reported to have strong lytic activity and high codon usage bias against their host (Bailly-Bechet *et al.*, 2007). In addition, the presence of tRNA is usually associated with the ability of phages to infect more hosts and have large burst size (Delesalle *et al.*, 2016). These are thus in perfect agreement with the broad host range of the phage vB_EcoM_11B2 results observed in this study (Chapter 4), which showed that this phage was capable of infecting three *E. coli* (O177, O157 and O26) serotypes from two different groups. This suggest that the phage vB_EcoM_11B2 is suitable for biocontrol application.

The *Escherichia* phage vB_EcoM_11B2-MVA genome annotation revealed no prophage repressor, lysogeny (integrase), virulence and antimicrobial related genes present. Furthermore, RAST and PATRIC annotation results revealed that the *Escherichia* phage vB_EcoM_11B2-MVA genome contained genes encoding for phage capsid and tail fibre proteins. *Escherichia* phage vB_EcoM_11B2-MVA genome harboured α-glycosyltransferase and anti-restriction nuclease encoding gene sequences. The role of α-glycosyltransferase in phage genome is to protect phage genome against host endonucleases during phage genome entry into the host cell (Markine-Goriaynoff *et al.*, 2004). In addition, phage anti-restriction nuclease plays a critical role in protecting the phage genome from host cell's restriction-modifying enzymes such as DNase or the CRISPER system (Louwen *et al.*, 2014). Other genes

encoding for phage tail lysozyme, phage T4-like protein, phage anti-restriction nuclease, DNA ligase and T4-like phage baseplate were found. The tail lysozyme was also found in the *Escherichia* phage vB_EcoM_Schickermooser genome (Korf *et al.*, 2019). These findings coupled with the other similar features found in *Escherichia* phage vB_EcoM_11B2-MVA genome, suggest that vB_EcoM_11B2 is safe to be used as a biocontrol agent or for phage therapy application, particularly in live animal.

Although ICTV employs many phage characteristics such as morphology and physiochemical properties and proteomics based methods, whole genome sequence is conserved to be the "gold standard" for taxonomic classification of phages (Ackermann, 2007). Several bioinformatics tools have been used to analyse phage genomes and thus facilitate their classification as per ICTV guideline (Richter *et al.*, 2015; Meier-Kolthoff and Göker, 2017). Despite the fact that 221 *Escherichia* phage genomes have been reported in the NCBI database, 26 (11.8%) are unclassified (accessed on 04, November 2019) (https://www.ncbi.nlm.nih.gov/Taxonomy). The VICTOR is a standalone method, which is used for classification of phages (Meier-Kolthoff and Göker, 2017). The method yields nucleotide or amino acid based phylogenies, showing high agreement with the ICTV classification criteria and thus allowing an informed taxonomic decision (Meier-Kolthoff and Göker, 2017; Korf *et al.*, 2019).

In this study, BLASTn and pair-wise alignment was performed to determine nucleotide similarities of the newly isolated genome and the other two phage genomes (*Escherichia* phage vB_EcoM_Schickermooser and *Escherichia* phage phAPEC8). Based on BLASTn analysis, the *Escherichia* phage vB_EcoM_11B2-MVA and *Escherichia* phage vB_EcoM_Schickermooser genomes revealed 99.02% homology and 95% alignment coverage. In addition, pair-wise comparison showed a linear dot plotting line with few gaps,

which indicated that the two genomes contained similar nucleotide sequences. On the other hand, dotplot analysis indicated more gaps between *Escherichia* phage vB_EcoM_11B2-MVA and *Escherichia* phage phAPEC8. This suggests that there is a difference between the nucleotides of the two genomes. Therefore, the *Escherichia* phage vB_EcoM_11B2-MVA genome is more similar to the *Escherichia* phage vB_EcoM_Schickermooser genome. The *in silico* JSpeciesWS analysis revealed that the newly isolated genome has high (ANIb > 96.33%) nucleotide identity similarities compared to the *Escherichia* phage genome, belonging to *Myoviridae* family.

The VICTOR and phylogenetic analysis clustered the *Escherichia* phage vB_EcoM_11B2-MVA genome with the *Escherichia* phage vB_EcoM_Schickermooser and thus showed high agreement with ICTV (Korf *et al.*, 2019). These strongly suggest that the *Escherichia* vB_EcoM_11B2-MVA genome belongs to Caudovirales, *Myoviridae* family. Given the fact that *Escherichia* phage vB_EcoM_Schickermooser belongs to an unknown species, a proposal has been submitted to ICTV to classify it under new genus, *Phapecoctavirus* (Korf *et al.*, 2019). The results obtained herein showed high similarities between the *Escherichia* phage vB_EcoM_11B2-MVA genome and the *Escherichia* phage vB_EcoM_Schickermooser genome, both at genome and nucleotide levels. Given that the *Escherichia* vB_EcoM_11B2-MVA genome is highly similar to the *Escherichia* phage vB_EcoM_Schickermooser genome, it is thus regarded as a new member of the "*Phapecoctavirus*" genus (not yet approved) (Korf *et al.*, 2019).

8.5. Conclusion

To the best our knowledge, this is the first study to perform a complete genome sequence of the *E. coli* O177-specific phage. Whole genome sequence analysis revealed that *Escherichia* vB_EcoM_11B2-MVA genome did not contained undesirable traits (lysogenic, virulence and

antimicrobial gene sequences). Furthermore, phage genome showed high identity similarities, especially with the *Escherichia* phage vB_EcoM_Schickermooser genome and thus classified under the order Caudovirales, *Myoviridae* family and "*Phapecoctavirus*" genus. Based on WGS results, the vB_EcoM_11B2-MVA phage is considered as a safe and suitable candidate to be used as a biocontrol agent against the *E. coli* O177 in live animals and food.

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CHAPTER NINE

GENERAL DISCUSSION, LIMITATIONS, CONCLUSION AND RECOMMENDATIONS

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GENERAL DISCUSSION, CONCLUSION, LIMITATIONS, RECOMMENDATIONS AND FUTURE WORK

9.1. General discussion

Atypical enteropathogenic *E. coli* (aEPEC) strains are emerging pathogens, which cause fatal diarrhoea infections in human worldwide (Ingle *et al.*, 2016; Xu *et al.*, 2017). Although this group is characterised by a lack of the *bfp* and *stx* operon, some strains may possess virulence factors of other diarrhoeagenic *E. coli* and extraintestinal pathogenic *E. coli* pathotypes (Trabulsi *et al.*, 2002; Gomes *et al.*, 2016). Previously, attempts were made to identify either novel or known genes to determine pathogenicity of aEPEC strains and the results indicated that this group harbours *stx* genes (Ingle *et al.*, 2016). However, these studies were limited to known shiga toxin producing *E. coli* strains such as O157 and non-O157 (Ateba and Mbewe, 2011; Wang *et al.*, 2013; Toro *et al.*, 2017). There is, therefore, limited information on virulence properties and antimicrobial resistance profiles of aEPEC, especially the O177 strain. As a result, this may pose a serious problem should there be an outbreak of aEPEC infections. Given that there is a limited number of novel antibiotics that can combat antimicrobial-resistant pathogens (Altamirano and Barr, 2019), there is a need to develop a novel and practical biocontrol interventions such as bacteriophages to mitigate antimicrobial resistance. Therefore, the aim of this study was to isolate and develop bacteriophage cocktails to reduce *E. coli* O177 in live cattle, using the *in vitro* rumen simulation model and on artificially contaminated beef.

In this study, the *E. coli* O177 strain was isolated from cattle faeces and the isolates were confirmed using the newly developed multiplex PCR analysis, targeting the *rmlB* and *wzy* genes. These are highly conserved genes located within the O-antigen cluster region (Beutin *et*

al., 2005). The specific primers and multiplex PCR assay designed in this study to detect the *E. coli* O177 strain were highly sensitive, rapid and reproducible. This demonstrates that these primers and the PCR assay could be used to discriminate and detect this strain from different sources. Although *stx* genes are commonly found in STEC strains (Kaper *et al.*, 2004), *E. coli* O177 strain isolated in this study harboured *stx*$_1$, *stx*$_2$, *stx*$_{2a}$, *stx*$_{2d}$, *hlyA* and *eaeA* genes. Given that *stx*$_2$, *stx*$_{2a}$, and *stx*$_{2d}$ genes are associated with HC and HUS infections in humans (Scheutz *et al.*, 2012), this indicates that *E. coli* O177 may cause similar symptoms in humans, resulting in serious public health implications.

Antimicrobial resistance is a major public health concern (Altamirano and Barr, 2019). The burden of antibiotic resistance is not only limited to high mortalities in humans and animals but it has serious financial costs accrued through efforts to contain the spread of the disease (Qiao *et al.*, 2018; Altamirano and Barr, 2019). Therefore, constant surveillance of antimicrobial resistant foodborne pathogens isolated from food producing animals and their associated food products is imperative. The current study revealed that *E. coli* O177 isolates were resistant to several antimicrobial agents from different groups of antibiotics. Interestingly, the isolates showed multi-drug resistant (MDR) and extensively drug-resistant (XDR) profiles. Patients with MDR or XDR infections are at high risk due to treatment failure (Altamirano and Barr, 2019). Furthermore, the *E. coli* O177 isolates harboured different antibiotic resistance gene determinants. Although few antibiotics were tested in Chapter 3 of this thesis, the importance of these findings cannot be overemphasised.

Genetic typing and whole genome sequencing (WGS) are useful techniques to determine the genetic profile of foodborne pathogens (Adzitey *et al.*, 2013; Ateba and Mbewe, 2014; Jagadeesan *et al.*, 2019). The data obtained using these techniques may provide genetic

similarities and virulence profiles of foodborne pathogens isolated from different sources and/or locations (Ateba and Mbewe, 2014). Genetic typing revealed that *E. coli* O177 isolates obtained from different farming systems shared genetic similarities. In addition, WGS showed that this strain harboured several virulence and antibiotic resistance genes. Interestingly, the *E. coli* O177 contained virulence genes similar to those found in other diarrhoeagenic *E. coli* and extraintestinal pathogenic *E. coli* pathotypes. This indicates that *E. coli* O177 is highly pathogenic, which is a major cause for concern for human health. The presence of virulence and antimicrobial resistance genes in *E. coli* O177 necessitates the development of novel interventions such as the use of bacteriophages to combat the problem of antibiotic resistance (Altamirano and Barr, 2019).

Lytic bacteriophages are considered to be viable alternatives to antibiotics in the fight against antimicrobial resistant foodborne pathogens to improve food safety (Doyle and Erickson, 2012; Spricigo *et al.*, 2013). Furthermore, lytic phages are capable of preventing biofilm formation by foodborne pathogens (Sadekuzzaman *et al.*, 2017; Yuan *et al.*, 2019). The lytic *E. coli* O177-specific phages isolated in this study had a wide host range, were stable over a wide range of pHs and remained viable at 40°C. Phages possessing these characteristics are considered to be most suitable for biocontrol purposes (Harada *et al.*, 2018). Interestingly, phages vB_EcoM_12A1 and vB_EcoM_3A1 and three cocktails, T3, T4 and T6, reduced *E. coli* O177 cell counts to below detection limit (1.0 $\log_{10}$ CFU/mL) on artificially contaminated beef over a seven-day period. Furthermore, these phages prevented biofilm formation by the *E. coli* O177 isolates. It is also worth mentioning that phage cocktails were more effective in destroying established biofilms within a five-hour incubation period compared to individual phages. This demonstrate that phage cocktails designed in this study have potential to prevent bacterial growth on meat under storage conditions as well as prevent biofilm formation.

Given that cattle harbour different foodborne pathogens, food contamination may occur during milking and slaughtering processes (Soon *et al.*, 2011; Baker *et al.*, 2016). This suggests that pre-harvest interventions that reduce pathogen load in live animals could be effective in minimising food contamination (Goodridge and Bisha, 2011). Although several strategies have been employed to mitigate foodborne pathogens in food, their side effects on both human and animals present a significant drawback (Sillankorva *et al.*, 2012). As a result, bacteriophages have been proposed to replace current intervention strategies with the intention of reducing the level of foodborne pathogens, especially in live animals (Goodridge and Bisha, 2011; Sillankorva *et al.*, 2012; Cooper, 2016). Although *E. coli* is commonly found in the lower part of the gastrointestinal tract (GIT) of cattle (Grauke *et al.*, 2002), pre-starvation (before slaughter) processes may precipitate proliferation of *E. coli* species in the rumen (Laven *et al.*, 2003). Furthermore, animals fed on high concentrates, especially at feedlots and dairy may suffer from subacute rumen acidosis, which may favour proliferation of *E. coli* species in the rumen (Tkalcic *et al.*, 2000; Khafipour *et al.*, 2011). As a result, this may increase bacterial population and thus cause meat contamination at slaughter.

A study was therefore, designed to evaluate the viability of phages and their potency against *E. coli* cells under a rumen-simulated environment. The results indicated that individual phages were able to reduce *E. coli* cell counts in that rumen simulated environment. Phage cocktails showed a relatively high (62.35 to 66.92%) relative reduction of *E. coli* O177 cell count than individual phages (61.02 to 62.74%). Phage cocktails T3, T4 and T6 were the most effective (66.67, 66.92 and 66.42, respectively) settings over 48 hours of incubation. This indicated that phage cocktails designed in this study could be used to reduce *E. coli* load in milking cows or before slaughter in feedlot cattle.

Although phages are considered to be a viable tool to deal with antimicrobial resistant pathogens, complete genome sequence of phages is required to avoid lysogenic phages, which may harbour undesirable traits (Fogg *et al.*, 2011). Whole genome sequencing provides novel insights into taxonomic classification and genetic features of phage genomes (Korf *et al.*, 2019). Even though the taxonomic classification of phage genomes has improved, some phage genomes remain unclassified (Korf *et al.*, 2019). Furthermore, genome information for lytic phages against non-O157 STEC and aEPEC is very rare (Liao *et al.*, 2019). This may affect full utilisation of phages against such strains. Therefore, this thesis ends by reporting the complete genome sequence of the *Escherichia* phage vB_EcoM_11B2.

The selection was based on the fact that, this phage had a high lytic activity, broad host range (*E. coli* O177, O157 and O26), and stable over a wide range of pHs and temperatures tested in the study. Genome analysis revealed that the *Escherichia* vB_EcoM_11B2 genome belongs to the *Myoviridae* family under the new "*Phapecoctavirus*" genus (not yet approved) (Korf *et al.*, 2019). Furthermore, the *Escherichia* vB_EcoM_11B2 genome possesses the holin trait, which is responsible for lytic activity. Furthermore, the genome does not harbour lysogenic (integrase), virulence and antibiotic resistance gene sequences. This confirms that *Escherichia* phage vB_EcoM_11B2 is safe and suitable for use in food or live animals.

9.2. Conclusion

This study reports, for the first time, the detection of an atypical enteropathogenic *E. coli* O177 strain and isolation of its specific lytic bacteriophages from cattle faeces in South Africa. The isolated *E. coli* O177 strain harbours several virulence genes, possesses antibiotic resistance genes and was found to be resistant to several antimicrobial agents. A high genetic similarity between the *E. coli* O177 isolates obtained from cattle faeces in different farming systems was

observed. *Escherichia coli* O177-specific phages revealed a high lytic activity against the *E. coli* O177, O26 and O157. In addition, phages were stable over a wide range of pH and at body temperature. Phage cocktails were more effective in reducing *E. coli* O177 cells on artificially contaminated beef than individual phages. Phages prevented biofilm formation by the *E. coli* O177 strain while also destroying pre-formed biofilms. In a simulated rumen fermentation system, phages were effective in reducing *E. coli* O177 cells with cocktails being more potent than individual phages. Complete genome sequence showed that a novel *Escherichia* vB_EcoM_11B2-MVA genome does not contain undesirable traits and, therefore, *Escherichia* vB_EcoM_11B2 is a safe and suitable candidate for phage therapy.

9.3. Limitations, Recommendations and Future Work

Although the aim of this study was achieved, the limitation of this study was the low number of antimicrobial agents tested for resistance against *E. coli* O177 isolates. Furthermore, only one lytic phage genome was sequenced in this study. However, this work can then be used as a baseline data for further studies. Future studies should be carried out to isolate the *E. coli* O177 strain from different sources such as food derived from food-producing animals, abattoirs and butcheries to establish other sources of the strain and its route of transmission. *In vivo* evaluations of the phages and their cocktails need to be carried out for validation. In addition, complete genome sequencing of *E. coli* lytic phages is required to expand our knowledge on the potential of phages in reducing foodborne pathogen in food and live animals. This will increase the number of phage candidates for phage therapy.

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APPENDICES

APPENDIX 1. ETHICS CERTIFICATE

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Date: 18/10/2019

To: Mr PK Montso

RE: Approval of your application by the FNAS Ethics Committee

Ethics number: NWU-01223-19-S9

Study title: Development of bacteriophage cocktail for bio-control of atypical Escherichia coli 0177 strains

Study leader: PROF CN ATEBA

Student: Mr PK Montso

You are kindly informed that after review by the FNAS Ethics committee, North-West University, your ethics approval application has been successful.

Your study has been approved as a **No Risk** project.

Yours sincerely,

Prof. Oriel Thekisoe

Acting Chairperson

FNAS Ethics Committee

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APPENDIX 2: LANGUAGE EDITING CERTIFICATE

DEPARTMENT OF BOTANY

Tel: +27 18 389 2289 Fax: 018 389 2134

Cell: +27 72 626 8446 E-mail: Oziniel.Ruzvidzo@nwu.ac.za

Date: 02nd December, 2019

To Whom It May Concern,

REF: Language Editing and Proof-reading of Dissertations/Theses

Dear Sir or Madam,

This serves to confirm that I have proof-read and edited the doctoral thesis of **KP Montso** (21261660; **orcid.org/0000-0002-3344-1270**) entitled: **Development of bacteriophage cocktail for bio-control of atypical *Escherichia coli* O177 strains.** The candidate then later corrected all the identified language and technical errors to my and the supervisor's utmost satisfaction. Thus the document presented here is of sufficient and acceptable academic standards.

Editor

Prof. O Ruzvidzo

Supervisor

Prof. CN Aleba

APPENDIX 3: PUBLICATION (Paper I)

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 24 September 2019
doi: 10.3389/fcimb.2019.00333

The First Isolation and Molecular Characterization of Shiga Toxin-Producing Virulent Multi-Drug Resistant Atypical Enteropathogenic *Escherichia coli* O177 Serogroup From South African Cattle

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Atypical enteropathogenic *E. coli* (aEPEC) is a group of diarrhoeagenic *Escherichia coli* with high diversity of serogroups, which lack the bundle-forming pili (BFP) and genes encoding for shiga toxins. The aim of this study was to isolate, identify and determine virulence and antibiotic resistance profiles of aEPEC O177 strains from cattle feces. A total of 780 samples were collected from beef and dairy cattle and analyzed for the presence of *E. coli* O177. One thousand two hundred and seventy-two (1272) presumptive isolates were obtained and 915 were confirmed as *E. coli* species. Three hundred and seventy-six isolates were positively confirmed as *E. coli* O177 through amplification of *rmlB* and *wzy* gene sequences using multiplex PCR. None of these isolates harbored *bfpA* gene. A larger proportion (12.74%) of the isolates harbored *hlyA* gene while 11.20, 9.07, 7.25, 2.60, and 0.63% possessed stx_2, stx_1, *eaeA*, stx_{2a}, and stx_{2d}, respectively. Most of *E. coli* O177 isolates carried stx_2/*hlyA* (9.74%). Furthermore, 7.40% of the isolates harbored stx_1/stx_2 while 7.09% possessed stx_1/stx_2/*hlyA* genes. Only one isolate harbored stx_1/stx_2/*hly*/*eaeA*/stx_{2a}/stx_{2d} while 5.11% of the isolates harbored all the four major virulence genes stx_1/stx_2/*hlyA*/*eaeA*, simultaneously. Further analysis revealed that the isolates displayed varied antimicrobial resistance to erythromycin (63.84%), ampicillin (21.54%), tetracycline (13.37%), streptomycin (17.01%), kanamycin (2.42%), chloramphenicol (1.97%), and norfloxacin (1.40%). Moreover, 20.7% of the isolates exhibited different phenotypic multi-drug resistance patterns. All 73 isolates harbored at least one antimicrobial resistance gene. The *aadA*, *streA*, *streB*, *erm*, and *tetA* resistance genes were detected separately and/or concurrently. In conclusion, our findings indicate that environmental isolates of aEPEC

APPENDIX 4: PUBLICATION (Paper II)

frontiers in Public Health

ORIGINAL RESEARCH
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Characterization of Lytic Bacteriophages Infecting Multidrug-Resistant Shiga Toxigenic Atypical *Escherichia coli* O177 Strains Isolated From Cattle Feces

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The increasing incidence of antibiotic resistance and emergence of virulent bacterial pathogens, coupled with a lack of new effective antibiotics, has reignited interest in the use of lytic bacteriophage therapy. The aim of this study was to characterize lytic *Escherichia coli* O177-specific bacteriophages isolated from cattle feces to determine their potential application as biocontrol agents. A total of 31 lytic *E. coli* O177-specific bacteriophages were isolated. A large proportion (71%) of these phage isolates produced large plaques while 29% produced small plaques on 0.3% soft agar. Based on different plaque morphologies and clarity and size of plaques, eight phages were selected for further analyses. Spot test and efficiency of plating (EOP) analyses were performed to determine the host range for selected phages. Phage morphotype and growth were analyzed using transmission electron microscopy and the one-step growth curve method. Phages were also assessed for thermal and pH stability. The spot test revealed that all selected phages were capable of infecting different environmental *E. coli* strains. However, none of the phages infected American Type Culture Collection (ATCC) and environmental *Salmonella* strains. Furthermore, EOP analysis (range: 0.1–1.0) showed that phages were capable of infecting a wide range of *E. coli* isolates. Selected phage isolates had a similar morphotype (an icosahedral head and a contractile tail) and were classified under the order Caudovirales, *Myoviridae* family. The icosahedral heads ranged from 81.2 to 110.77 nm, while the contractile tails ranged from 115.55 to 132.57 nm in size. The phages were found to be still active after 60 min of incubation at 37 and 40°C. Incremental levels of pH induced a quadratic response on stability of all phages. The pH optima for all eight phages ranged between 7.6 and 8.0, while at pH 3.0 all phages were inactive. Phage latent period ranged between 15 and 25 min while burst size ranged from 91 to 522 virion particles [plaque-forming unit (PFU)] per infected cell. These results demonstrate that lytic *E. coli* O177-specific bacteriophages isolated from cattle feces are highly stable and have the capacity to infect different *E. coli* strains, traits that make them potential biocontrol agents.

Keywords: atypical enterophagenic *E. coli* O177, bacteriophages, bacteriophage therapy, biocontrol, biological properties, multi-drug resistance, shiga-toxigenic *E. coli*