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## FACULTY OF VETERINARY SCIENCE

## DEPARTMENT OF PARACLINICAL SCIENCES

## VETERINARY PUBLIC HEALTH SECTION

**Induction of lysogenic bacteriophages of Shiga toxin-producing *Escherichia coli* by antimicrobial growth promoters used in food-producing animals in South Africa.**

**By**

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**A dissertation submitted in partial fulfilment of the requirements for the degree of Master of Science (Veterinary Science)**

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# DEDICATION

First and foremost, I would like to thank God for granting me such an opportunity to study and for giving me the strength to never give up when times were hard. A lot has happened during the course of my study but He assured me that the good work that He had begun in my life He would finish and surely that came to pass. I dedicate this degree to Him just because He has done it for me. Without Him I would not be where I am right now, therefore I am forever grateful.

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# DECLARATION

This dissertation is my original work and has not been presented for any award or degree in another University.

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# THESIS SUMMARY

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 is a foodborne disease characterized by diarrhoea and complications such as haemorrhagic colitis and the haemolytic uremic syndrome, a complication which can lead to kidney failure in 5-10% of humans showing STEC disease. The major virulence factors of STEC are two toxins (Stx1 and Stx2) encoded on bacteriophages, commonly termed Shiga toxin-converting bacteriophages (*stx*-converting bacteriophages). Previous studies have shown that a number of antimicrobials which are used for livestock growth promotion can induce *stx*-converting bacteriophages at subinhibitory concentrations. Induced *stx*-converting bacteriophages are considered the main "drivers" of STEC emergence and evolution as they transfer bacteriophage-encoded Shiga toxin-encoding genes from STEC to naïve *E. coli* by transduction. This phenomenon is considered to be behind the formation of novel STEC strains. Although a European Union-wide ban on the use of antimicrobial growth promoters in animal agriculture exists since 2006, this controversial practice remains in effect in a number of countries around the world including South Africa. Therefore, in this study, four antimicrobials which are approved for livestock growth-promotion in South Africa were tested at sub-inhibitory concentrations for their capacity to induce Shiga toxin-converting bacteriophages from 47 STEC O157:H7 isolates using the double-layer agar-plaque assay. The antimicrobials tested included josamycin, virginiamycin, flavophospholipol and poly 2-propenal 2-propenoic acid. These antimicrobial growth promoters had never been tested for their capacity to induce bacteriophages. In addition, induced bacteriophages were characterized for the presence of genes encoding different Shiga toxin subtypes (*stx1, stx2, stx2c* and *stx2d*), restriction fragment length polymorphisms and morphology by electron microscopy.

The following bacteriophage induction rates were obtained for each antimicrobial growth promoter tested: poly 2-propenal 2-propenoic acid, 42.6% (20/47); virginiamycin, 34.0% (16/47); josamycin, 34.0% (16/47); flavophospholipol, 29.8% (14/47). A small number of STEC O157:H7 isolates induced bacteriophages spontaneously (14.9%; 7/47). Most of the induced bacteriophages carried the *stx2* and *stx2c*-encoding genes, independent of the induction method while only a few bacteriophages carried *stx2d* except for josamycin and spontaneously-induced bacteriophages which *stx2d* at higher rates of 87.5% (14/16) and 100% (7/7). Electron microscopy revealed only four representative groups of virion particle morphologies: three groups of bacteriophages with either a long hexagonal, oval/circular, and elongated head which all had long tails and one group of bacteriophages with an icosahedral/hexagonal head and a thick contractile tail. These results showed that josamycin, virginiamycin, flavophospholipol and poly 2-propenal 2-propenoic acid induce *stx*-converting bacteriophages from STEC O157:H7. Induced bacteriophages were largely *stx2* and *stx2c* positive, but *stx2d* positive to a lesser extent. Induction of *stx*-converting bacteriophages by antimicrobial growth promoters may be contributing to the conversion of naïve *E. coli* into virulent STEC strains as a result of bacteriophage transduction and horizontal transfer of *stx*-encoding genes from STEC to naive *E. coli*. This phenomenon has been identified as the driving force behind STEC emergence, expansion and evolution. The formulation of policies and implementation of strategies which promote the prudent use of antimicrobials growth promoters in animal agriculture in South Africa and elsewhere where these compounds are still in use are recommended.

# LIST OF ABBREVIATIONS

**AE:** Attaching and effacing

**HCL**: Hydrochloric acid

***E. coli***: *Escherichia coli*

**EDTA**: Ethylenediaminetetraacetic acid

**EFSA**: European Food Safety Authority

**LB**: Luria-Bertani

**LEE**: Locus of enterocyte effacement

**HC**: Haemorrhagic colitis

**HUS**: Haemolytic-uremic syndrome

**MIC**: Minimum inhibitory concentration

**mPCR**: Multiplex polymerase chain reaction

**Nle**: Non-LEE encoded effectors

**OI**: O-Island

**ORF**: Open reading frame

**PAI**: Pathogenicity Island

**PCR**: Polymerase chain reaction

**pPPA**: Poly 2-propenal 2-propenoic acid

**RFLP**: Restriction fragment Length Polymorphism

**SIC**: Subinhibitory concentration

**STEC**: Shiga toxin-producing *Escherichia coli*

**T3SS**: Type III secretion system

**Tir**: Translocated intimin receptor

**UV**: Ultraviolet

**VTEC**: Verocytoxin/verotoxin-producing *Escherichia coli*

**STEC**:Shiga toxin-producing *Escherichia coli*

**Stx**: Shiga toxin

**UV**: Ultra-violet light

# 1.0 CHAPTER I

# GENERAL INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC), also known as verocytotoxin-producing *E. coli* (VTEC), is a food-borne pathogen commonly associated with disease characterised by mild watery to severe bloody diarrhoea, haemorrhagic colitis and the haemolytic uremic syndrome (HUS). HUS remains the main human STEC disease complication. HUS is characterized by microangiopathy, haemolytic anaemia, thrombocytopenia, and often leads to acute renal failure, particularly in children and the elderly (Karmali *et al.* 1983; Joseph *et al.* 2020).

Ruminants, particularly cattle, sheep and goats are the natural reservoirs of STEC (Gyles 2007). Humans acquire STEC infections by ingestion of food or water contaminated with faeces containing this pathogen. STEC can also be transmitted from person-to-person or through contact with infected animals or the environment (Kintz *et al.* 2017; Reynolds *et al.* 2020).

More than 1000 STEC serotypes have been isolated from humans, domestic livestock and wild animals, food, and the environment (Bettelheim 2007; Beutin and Fach 2014; Bettelheim and Goldwater 2019). STEC O157:H7 was the first serotype to be associated with human disease (Riley *et al.* 1983; Wells *et al.* 1983). Furthermore, STEC O157:H7 remains the dominant and most investigated serotype. However, non-O157 STEC serogroups are commonly implicated in sporadic human illnesses and outbreaks worldwide and may be responsible for up to 80% of STEC infections in humans (Brooks *et al.* 2005; Johnson *et al.* 2006; Bettelheim and Goldwater 2019). Non-O157 STEC serogroups including O26, O45, O103, O121, O111 and O145 are considered the serogroups that are most frequently associated with human disease (Bettelheim and Goldwater 2019; Panel *et al.* 2020). Together with STEC O157:H7,

STEC O26, O45, O103, O121, O111 and O145 are commonly termed the “Big 7” or “Top 7” STEC (Beutin and Fach 2014).

The most important virulence factors of STEC are two Shiga toxins (also termed verocytotoxins or verotoxins) including Stx1 and Stx2. Shiga toxin-encoding genes are carried on the genome of lysogenic lambdoid bacteriophages (prophages) which are integrated in the chromosome of STEC (Konowalchuk *et al.* 1977; O'Brien *et al.* 1983; O'Brien *et al.* 1984; Newland *et al.* 1985; Huang *et al.* 1986; Strockbine *et al.* 1986; Smith and Scotland 1988; Strockbine *et al.* 1988; Scheutz *et al.* 2012).

Other main virulence factors include intimin (*eaeA)* (McDaniel *et al.* 1995b; Tzipori *et al.* 1995a) and haemolysin (*ehxA* or *hlyA*) (Schmidt *et al.* 1995). Intimin is an adhesin encoded on the locus of enterocyte effacement (LEE) pathogenicity island (McDaniel *et al.* 1995a; Tzipori *et al.* 1995b). Intimin is responsible for the formation of the typical attaching and effacing (AE) lesion observed in the intestinal epithelium of hosts infected with *eaeA* positive STEC (McDaniel *et al.* 1995a; Tzipori *et al.* 1995b; McDaniel and Kaper 1997). The plasmid-encoded haemolysin (*hlyA*) is a pore-forming toxin responsible for lysis of human erythrocytes and subsequent release of iron from haeme (Beutin *et al.* 1989; Schmidt *et al.* 1995; Schmidt and Karch 1996). The availability of iron in the host amplifies the multiplication and survival of STEC (Schmidt *et al.* 1995; Schmidt and Karch 1996). Furthermore, a number of proteins which are encoded on genes located on the LEE and other pathogenicity islands (PAIs) (non-LEE-encoded effector proteins) are also considered STEC virulence markers (Gruenheid *et al.* 2004; Mundy *et al.* 2004; Tobe *et al.* 2006; Coombes *et al.* 2008; Wong *et al.* 2011).

Current data shows that STEC is responsible for human enteric infections globally (Majowicz *et al.* 2014). The European Centre for Disease Prevention and Control,

reported that STEC was responsible for 6534-8658 cases of human STEC infections between 2018 and 2022 in 9 European Union/European Economic Area countries (https://www.ecdc.europa.eu/sites/default/files/documents/STEC_AER_2022_Report.pdf). Majowicz et al. have estimated that STEC accounts for 2,801,000 acute illnesses, 3,600 hospitalizations and 3890 cases of HUS per year globally (Majowicz *et al.* 2014). STEC has also been incriminated in human enteric illness in Africa including South Africa (Effler *et al.* 2001; Karama *et al.* 2019).

Although a large body of research on STEC is available since its first occurrence in 1982 (Riley *et al.* 1983), gaps still exist in understanding how STEC have emerged as pathogens. Research has shown that a number of antimicrobials which are used for livestock growth promotion can induce *stx*-converting bacteriophages (Kohler *et al.* 2000; Kim *et al.* 2016). Some reports have also found that when *stx*-converting bacteriophages are induced and released from STEC, they are able to transduce and transfer *stx*-encoding genes from STEC to naïve *E. coli* (Bielaszewska et al. 2007). Transfer of *stx*-encoding genes to naïve *E. coli* by transduction is the phenomenon responsible for the evolution, emergence and spread of new STEC strains (Allison 2007).

Therefore, the current study tested the capacity of antimicrobials approved for livestock growth promotion in South Africa to induce *stx*-converting bacteriophages from a collection of STEC O157:H7 isolates. Antimicrobials which were used to induce *stx*-converting bacteriophages included virginiamycin, josamycin, flavophospholipol and poly 2-propenal 2-propenoic acid. These compounds were banned in the European Union in 2006 (Phillips 2007), making their use in animal agriculture a controversial practice. Moreover, virginiamycin, josamycin, flavophospholipol and poly

2-propenal 2-propenoic acid have never been tested/assessed for their capacity to induce bacteriophages.

### 1.1 Aim and Objectives

The overall aim of this research was to investigate the effect of antimicrobial growth promoters on *stx*-converting bacteriophages induction from STEC O157:H7 isolates.

### 1.2 Specific objectives were to:

1) Determine the capacity of four antimicrobial growth promoters (virginiamycin, josamycin, flavophospholipol and poly 2-propenal 2-propenoic acid) which are approved for use in South African animal agriculture, to induce lysogenic bacteriophages from STEC O157:H7.
2) Characterize induced *stx*-converting bacteriophages with respect to the presence of *stx* subtypes, structural genes, restriction fragment length polymorphisms profiles, genome size and morphology.

The ultimate and long-term goal was to stimulate discussions which may lead to the formulation of new policies aimed at promoting the prudent use of antimicrobial growth promoters in animal agriculture.

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# 2.0 CHAPTER II

# LITERATURE REVIEW

## 2.1 Background

Shiga toxin-producing *E. coli* (STEC) also termed verotoxin-producing *E. coli* (VTEC) is a foodborne pathogen which causes human disease characterized by abdominal cramps, mild to severe diarrhoea, haemorrhagic colitis (HC) and complications including haemolytic uremic syndrome (HUS), a leading cause of acute kidney failure around the world (Kaper and Karmali 2008). Estimates show that STEC may be responsible for 2,801,000 cases of acute diseases annually and 3890 cases of HUS, worldwide (Majowicz *et al.* 2014). Human STEC infections are acquired by ingestion of food of animal origin with meat and in particular beef as well as dairy products reported as the predominant sources of transmission to humans (Beutin and Fach 2015). In addition, water and raw vegetables that are contaminated with cattle faeces are also considered sources of foodborne STEC for humans (Farrokh *et al.* 2013; Feng 2014; Beutin and Fach 2015; Saxena *et al.* 2015).

The main animal reservoirs of STEC are domestic ruminants including cattle, sheep and goats (Hussein and Bollinger 2005; Hussein and Sakuma 2005; Gyles 2007; Persad and Lejeune 2015; Mainga *et al.* 2018; McCarthy *et al.* 2021; Malahlela *et al.* 2022). These animal reservoirs may be spillover hosts, transmitting the pathogen without maintaining it; or dead-end hosts which transmit STEC solely by contaminated food (Gonzalez and Cerqueira 2020).

STEC exist as thousands of genetic variants and more than 1000 serotypes have been identified (Beutin and Fach 2014; Panel EFSA BIOHAZ *et al.* 2020). Most human STEC infections have been associated with serotype O157:H7. However, six non-

O157 STEC serogroups including O26, O45, O103, O111, O121 and O145 are also frequently incriminated in outbreaks of human disease, accounting for up to 80% of all human STEC infections (Brooks *et al.* 2005; Johnson *et al.* 2006). These six STEC serogroups which are frequently incriminated in human disease outbreaks, together with STEC O157 are commonly termed "Top 7/Big 7" STEC. However, a recent European Union report concluded that all STEC are infectious and able to cause mild to severe disease including HUS in humans (Panel EFSA BIOHAZ *et al.* 2020).

STEC strains were first linked to HUS in 1983 (Karmali *et al.* 1983), though HUS had been known and associated with acute renal failure, thrombocytopenia and microangiopathic anaemia since 1955 (Gasser 1955). Initially, it was postulated that HUS could be due to a viral or bacterial infection. Then *S. dysenteriae* was suspected to be a cause of HUS despite faecal samples collected from HUS patients yielding generic *E. coli* but no pathogenic bacteria. In 1968, in a survey on HUS in South Africa, it was postulated that HUS may have been caused by a mutagenic "*E. coli* strain" carrying a lysogenic bacteriophage (Kiibel and Barnard 1968). In 1983, STEC was associated with *E. coli* O157:H7 carrying lysogenic Shiga toxin-encoding bacteriophages (Smith and Scotland 1988). Later, induction of Shiga toxin-encoding bacteriophages from STEC was demonstrated (O'Brien *et al.* 1983).

The first association of STEC O157:H7 with a case of human haemorrhagic colitis disease in South Africa was in 1990 (Browning *et al.* 1990). Later, in 1992, a large outbreak of STEC O157 hemorrhagic colitis occurred from October through November along the border of South Africa and Eswatini (Isaacson *et al.* 1993; Armstrong *et al.* 1996). The outbreak affected many people in Eswatini, and Mpumalanga and KwaZulu-Natal provinces of South Africa (Isaäcson *et al.* 1993; Effler *et al.* 2001). The 1992 outbreak of bloody diarrhoea caused by STEC O157 in Southern Africa resulted

in 40,912 hospital visits (Effler et al., 2001). This outbreak occurred after a long drought and was linked to the consumption of water from a river which had been contaminated with faeces from dead cattle carcases (Effler *et al.* 2001).

## 2.2 Virulence factors of Shiga toxin-producing *Escherichia coli*

### 2.2.1 Shiga toxins

The main virulence factors of STEC are two Shiga toxins (Stx1 and Stx2) which belong to two distinct antigenic groups (Strockbine *et al.* 1986). Genes encoding Shiga toxin genes are located on the genome of lambdoid bacteriophages which are prophages integrated as lysogens into the STEC chromosome (Smith *et al.* 1983; O'Brien *et al.* 1984). *Stx2* is the toxin most commonly associated with severe STEC infections in humans (Ostroff *et al.* 1989; Boerlin *et al.* 1999). Epidemiological data have also linked STEC strains that encode Stx2 and Stx2c to more severe disease in comparison to strains that carry *stx1* only or both s*tx1* and *stx2* (Boerlin *et al*. 1999; Friedrich *et al.* 2002). Furthermore, five *stx1* (*stx1a, stx1b*, *stx1c*, *stx1d* and *stx1e*) and 13 for *stx2* (*stx2a*, *stx2b*, *stx2c*, *stx2d*, *stx2e*, *stx2f*, *stx2g, stx2h, stx2i, stx2j, stx2k, stxl and stx2m, stx2n, stx2o*) subtypes have been identified (Scheutz *et al.* 2012; Bai *et al.* 2018b; Bai *et al.* 2018a; Hughes *et al.* 2019; Panel *et al.* 2020; Gill *et al.* 2022; Lindsey *et al.* 2023). However, the roles played by *stx* variants other than *stx2*, *stx2c* and *stx2d* in human disease occurrence remain unknown or poorly understood.

### 2.2.2 Intimin

In addition to Shiga toxins, some STEC strains produce intimin (EaeA) which is located on the locus of enterocyte effacement (LEE). This is a pathogenicity island encoding effector proteins responsible for attaching and effacing (AE) lesions in the host

intestine infected with *eaeA*-positive STEC (McDaniel *et al.* 1995; Tzipori *et al.* 1995). LEE-encoded effector proteins are functionally under the control of a type III secretion system (T3SS), a complex molecular “needle or syringe”, which mainly regulates and facilitates the secretion of intimin (Eae) and its receptor the translocated intimin receptor (Tir) during STEC adhesion to host cells in the intestinal mucosa (Hacker and Kaper 2000). Intimin is commonly used as a marker (*eaeA*) of virulence in STEC. While the presence of *eaeA* in STEC strains has been associated with severe disease in humans (Panel EFSA BIOHAZ *et al.* 2020), it is not essential for disease causation, as *eaeA*-negative STEC are also incriminated in mild to severe enteric disease and HUS (US Department of Agriculture and Service 2012).

### 2.2.3 Plasmid encoded virulence markers

STEC strains possess large plasmids of variable sizes (Karch *et al.* 1998; Brunder *et al.* 1999). These plasmids encode a number of virulence factors (Perna *et al.* 2001) that exacerbate pathogenicity and survival of STEC in humans (Schmidt *et al.* 1995; Karch *et al.* 1998; Brunder *et al.* 1999). All STEC O157:H7 possess a large plasmid which has been termed pO157 (Karch *et al.* 1993; Karch *et al.* 1998). The following virulence markers are encoded on pO157: enterohemolysin (*ehxA*) (Schmidt *et al.* 1995), extracellular serine protease (*espP*) (Brunder *et al.* 1997), catalase-peroxidase (*katP*) (Brunder *et al.* 1996), and type II secretion pathway (*etpD*)(Schmidt *et al.* 1997). The *ehxA* gene encodes a heat labile pore-forming toxin that lyses human enterocytes with subsequent release of iron from haeme. The presence of this toxin in STEC strains has been associated with HUS and has been shown to enhance STEC multiplication and survival *in vivo* by availing iron to bacteria. Iron is a much-needed molecule for bacterial survival in the host when which becomes available when haeme

breaks down (Brunder *et al.* 1996; Schmidt and Karch 1996). Catalase-peroxidase (KatP) facilitates bacterial survival against oxidative damage caused by host phagocytes (Brunder *et al.* 1996). The type II secretion system (*etpD*) functions as a transmembrane pump that carries facilitates various proteins secretion pathways from the STEC O157:H7 to target host cells (Schmidt *et al.* 1997; Lory 1998). The extracellular serine protease (EspP) cleaves coagulation factor V responsible for gastrointestinal bleeding in hemorrhagic colitis (HC) (Brunder *et al.* 1997) and has been shown to play a role in STEC O157:H7 intestinal colonization in calves and adherence to intestinal epithelial cells in adult cattle (Dziva *et al.* 2007).

Additional plasmid-encoded virulence markers are the STEC autoagglutinating adhesin (*saa*) and subtilase cytotoxin (*subA*) (Paton *et al.* 2001a; Paton and Paton 2010). Saa is an adhesin responsible for adherence of *eaeA*-negative STEC to host epithelial cells (Paton *et al.* 2001a), whereas the subtilase cytotoxin contributes to STEC pathogenesis by suppressing the immune system and damaging tissues in the host (Paton *et al.* 2001a; Paton *et al.* 2004). Genes encoding both proteins have been found exclusively in *eaeA*-negative STEC strains (Paton *et al.* 2001b; Karama *et al.* 2019).

### 2.2.4 Pathogenicity islands encoded virulence factors

The whole genomes sequences of STEC O157:H7 EDL933 and Sakai strains revealed more than 170 pathogenicity Islands (PAIs) as genomic islands (O islands) carrying various virulence-associated genes (Perna *et al.* 1998; Hayashi *et al.* 2001). Genes located on pathogenicity islands (PAIs/O islands) namely, OI-36, OI-43/48, OI-57, OI-71 and OI-122 encode a number of virulence genes which enhance STEC pathogenicity (Perna *et al.* 2001; Taylor *et al.* 2002; Karmali *et al.* 2003; Shen *et al.*

2004; Coombes *et al.* 2008). These genes have been used in molecular risk assessment (MRA) investigations to predict and categorize STEC serotypes into various seropathotypes depending on the association of a particular serotype with human disease (Karmali et al., 2003, Coombes et al., 2008).

OI-43/48 carries *ureC* (Urease)*, terC* (Tellurite) and *iha* (IrgA homologue adhesion) are (Perna *et al.* 2001; Taylor *et al.* 2002; Yin *et al.* 2009). Urease facilitates the conversion of urea to ammonia which buffers and raises pH in the acidic environment of the host cell, which enhances and favours STEC survival by tolerating acid conditions in the host stomach and cells (Mobley *et al.* 1995; Nakano *et al.* 2001; Steyert *et al.* 2011; Steyert and Kaper 2012). Tellurite resistance (*ter*) assists in STEC response to stress conditions within any given host environment (Taylor *et al.* 2002). The IrgA homologue adhesin (*iha*) facilitates STEC attachment to intestinal epithelial cells (Tarr *et al.* 2000).

OI-122 carries at least four open reading frames (ORF) which are homologous to previously described virulence genes: *Z4326/espL2* (*sen*), *Z4333 (efa1*), *Z4332* (*efa1*) and *Z4321* (*pagC*) (Karmali *et al.* 2003). *Z4332* and *Z4333* share homology with enterohemorrhagic *E. coli* (EHEC) factor for adherence gene cluster *efa1* and *efa2* of STEC O157:H7. The *efa* gene encodes a protein that enables bacterial adherence and suppresses proliferation of host lymphocytes by increasing intestinal STEC colonization in cattle (Nicholls *et al.* 2000; Morabito *et al.* 2003; Konczy *et al.* 2008). ORF *Z4321* gene is homologous to *pho*P-activated gene C (*pagC*) found in *Salmonella* Typhimurium (Miller and Mekalanos 1990; Gunn *et al.* 1995) while *Z4326* is homologous to enterotoxin 2 (*sen)* found in *Shigella flexneri (Nataro et al. 1995)*. Both *pagC* and *sen* genes encode proteins which play a role in the modulation of bacterial virulence and enhance bacterial survival within host macrophages (Gunn *et al.* 1995;

Ju *et al.* 2013). Additional functions of proteins encoded on OI-122 include cell adhesion, immunosuppression, disruption of epithelial barrier function (Klapproth *et al.* 2000).

### 2.2.4.1 Non-LEE encoded effectors

Several pathogenicity islands carry gene sequences coding for numerous effector proteins which are located on the LEE pathogenicity island and are not under its control. Most of these effectors, which have been termed “non-LEE encoded effectors” (nles) (Coombes *et al.* 2008; Dean and Kenny 2009; Wong *et al.* 2011; Salvador *et al.* 2014), are often encoded on prophage modules on different pathogenicity islands (Perna *et al.* 1998). Non-LEE-encoded effector proteins are virulence-associated proteins which are delivered into the host cell during STEC infection. A number of functions have been ascribed to *nles* including blocking cell division, inhibition of apoptotic activities and phagocytosis, damage to the host innate immune response, disruption of microtubule cytoskeleton, and enhancing paracellular permeability (Dean and Kenny 2009; Wong *et al.* 2011; García-Angulo *et al.* 2012).

OI-36 PAI carries *nleB2 (Z0985), nleC (Z0986), nleD (Z0990)* and *nleH1-1 (Z0989)* genes which code for proteins which enhance STEC colonization of host intestinal mucosa and suppression of host immune system (Dziva *et al.* 2004; García-Angulo *et al.* 2008; Pearson *et al.* 2011). OI-57 carries *nleG6-2*, *nleG5-2* and *nleG2-3* (Coombes *et al.* 2008; Imamovic *et al.* 2010; Wu *et al.* 2010), while OI-71 carries *nle*A (Z6024), *nleF* (Z6020), *nleG* (*Z6010*), *nleG2-1* (*Z6025*), *nleG9* (*Z2560*) *nleH1-2* (*Z6021*) which play a role in maximizing STEC colonization and inhibiting host immune proteins (Gruenheid *et al.* 2004; Tobe *et al.* 2006). OI-122 carries *nleB* (*Z4328*), *nleE* (*Z4329*). The *nleB* and *nleE* genes encode proteins that regulate host cell functions,

suppressing host immune system through enhancement of STEC virulence and colonization and thus reducing the infective dose of STEC (Kelly *et al.* 2006; Wickham *et al.* 2006).

## 2.3 Shiga toxin-converting bacteriophages

### 2.3.1 Morphology of Shiga toxin-producing *Escherichia coli* bacteriophages

Although *stx*-converting bacteriophages exhibit considerable diversity (Johansen *et al.* 2001; Karama and Gyles 2008a; Krüger and Lucchesi 2015), their common feature is the head-tail morphology. Different families of *stx*-converting bacteriophages exhibit hexagonal, icosahedral, or elongated/prolate/oblong shapes. The tails are long or short, lack or possess tail fibres, and contractile or non-contractile (Karama and Gyles 2008b; Smith *et al.* 2012; Rodríguez-Rubio *et al.* 2021). Stx-converting bacteriophages are classified based on their morphological structures into different families of the order *Caudovirales* such as: *Podoviridae*, *Siphoviridae*, *and Myoviridae*. For example, most *stx2*-encoding bacteriophages are classified as belonging to the *Podoviridae* family with typical regular hexagonal heads and short tails (Muniesa *et al.* 2004; Smith *et al.* 2007; Smith *et al.* 2012). A prototype bacteriophage of the *Podoviridae* family is the *stx2a*-positive 933W phage, a lysogen of the *E.coli* O157:H7 strain EDL 933 (Plunkett III *et al.* 1999). *Siphoviridae* have elongated heads and a long non-contractile tail. A typical example is the *Stx1* H19-B bacteriophage (Huang *et al.* 1986; Muniesa and Schmidt 2014). *Myoviridae stx*-converting bacteriophages possessing icosahedral heads with long tails which may also be contractile have been less frequently described (Rooks *et al.* 2012).

### 2.3.2 Genome organisation of Shiga toxin-converting bacteriophages

Analysis of the genome sequences from two STEC O157:H7 strains (EDL933 and Sakai) (Perna *et al.* 1998; Hayashi *et al.* 2001) has revealed that both strains have 18 and 24 multigenic regions encoding intact or remnants of bacteriophages sequences. Notable differences between sequenced non-pathogenic *E. coli* K-12 strain and STEC have been ascribed to prophages sequences embedded in the *E. coli* chromosome (Blattner *et al.* 1997; Perna *et al.* 1998; Hayashi *et al.* 2001).

The genomic organization of Stx-encoding bacteriophages is similar to that of bacteriophage lambda and related lambdoid bacteriophages (Huang *et al.* 1987). These bacteriophages possess double stranded DNA (Campbell 1994; Casjens and Hendrix 2015) and are organized into early and late gene modules responsible for *stx*-bacteriophage replication, virion particle assembly and release after lysis of the host bacteria (Neely and Friedman 1998). Shiga toxin production is co-regulated with Stx bacteriophage induction and its release controlled by bacteriophage late genes (Fuchs *et al.* 1999). Late genes are only expressed under the phage lytic growth phase while no Stx is produced during the lysogenic state, as genes regulating Stx production are dormant until bacteriophage induction occurs.

Bacteriophages genes that encode Stx1 and Stx2 are organised into two conserved modules namely $stxA_1$ and $stxB_1$, under the control of the promoter *pR* and located in the late-phase region, flanked downstream by *R*, *Rz* and *S* genes encoding proteins responsible for bacteriophages lysis and upstream by *λ Q* antiterminator gene sequences, which regulate virion particle release (Unkmeir and Schmidt 2000). The *cI* repressor gene, the *cro* gene which regulates type III secretion in anaerobic environments and is involved in phage immunity, and the two genes *O* and *P* which

are responsible for phage transcription and replication initiation respectively, are conservatively found upstream of the *Q* antiterminator gene sequence (Neely and Friedman 1998; Schmidt 2001; Krüger and Lucchesi 2015).

In addition, a number of core functional genes including *CIII,* which encodes a protease inhibitor and *N1* and *N2* which encode late anti-terminator proteins, also play an important role in the prophage regulation (Latała *et al.* 2001; Smith *et al.* 2007). The *CIII* gene is responsible for the prophage state or lysogeny maintenance (Latala et al., 2001). During lysogeny, *cI* repressor genes are not expressed.

Another important bacteriophage structural gene is *IS1203/*Integrase which encodes integrase (Fogg *et al.* 2007). Stx-bacteriophages possess various chromosomal integration sites (Fogg *et al.* 2007). STEC O157:H7 has two principal integration sites including *wrbA* and *yehV* (Shaikh and Tarr 2003; Besser *et al.* 2007). Other integrations sites in STEC O157 serogroups are *yecE*, and *argW* (De Greve *et al.* 2002) and *sbcB* (Ohnishi *et al.* 2002). Non-O157 STEC have unique integration sites including *argW, prfC, potC, ssrA, serU, wrbA, yciD, yecE, yecD, ynfH, yjbM* and *Z2577* (Recktenwald and Schmidt 2002; Koch *et al.* 2003; Fogg *et al.* 2007).

Despite similarities in genome organisation among *stx*-converting bacteriophages and lambda phages, significant diversity exists between *stx*-converting and lambdoid bacteriophages such as those of genome sizes which can range from 28.7 to 71.9 kb (Krüger and Lucchesi 2015; Yang *et al.* 2020; Yara *et al.* 2020; Rodríguez-Rubio *et al.* 2021). Furthermore, there is diversity among *stx*-converting bacteriophages structural genes depending on the origin of the STEC O157:H7 isolates harbouring them, as previously shown in studies in comparative studies between *stx*-bacteriophages induced from fly versus cattle isolates (Ahmad and Zurek 2006) and clinical versus bovine STEC strains (Abedon and LeJeune 2005). Moreover, structural genes may

also vary depending on the subtype of the gene (Q21 vs *Q33*) or whether a particular gene subtype is truncated or complete (Teel *et al.* 2002). More recent studies reported undescribed novel sequences of *P* replication initiation genes of non-lambdoid *stx*-converting bacteriophages (Llarena *et al.* 2021).

### 2.3.3 Induction of *stx*-converting bacteriophages

Stx-converting bacteriophages and Shiga toxin production can be induced or may occur spontaneously under adverse environmental conditions when bacterial stressors/stimuli such as DNA-damaging compounds activate the bacteriophage SOS response system (Kimmitt *et al.* 2000; Kohler *et al.* 2000; Tarr *et al.* 2005; Allison 2007). The SOS DNA repair response or mechanism is a system controlled by two proteins; a repressor called LexA and an inducer, RecA. The LexA repressor inhibits transcription of SOS response genes by binding onto the promoter region of these genes. The RecA filament initiates the cleavage of LexA as soon there is DNA damage (Radman 1975). The expression of the RecA protein allows for derepression when the CI phage repressor is depleted, thus initiating transcription of late phage genes including *stx* genes which switches on bacteriophage induction, replication, assembly and release.

The following DNA-damaging agents or compounds including antimicrobials have been shown to induce *stx*-converting bacteriophages: ultraviolet light (O'Brien *et al.* 1984; O'Brien *et al.* 1989; Rietra *et al.* 1989; Osawa *et al.* 2000; Karama and Gyles 2008a; Łoś *et al.* 2009; Yue *et al.* 2012; Rahman *et al.* 2018; Zhang *et al.* 2020), mitomycin C (O'Brien *et al.* 1989; Johansen *et al.* 2001; Wagner *et al.* 2002; Muniesa *et al.* 2004; Zhang *et al.* 2020), various antimicrobials (Grif *et al.* 1998; Kimmitt *et al.* 2000; Kohler *et al.* 2000; Gamage *et al.* 2004; McGannon *et al.* 2010; Yan *et al.* 2011;

Ramstad *et al.* 2021), high temperatures/heat, hydrogen peroxide (Łoś *et al.* 2009; Yue *et al.* 2012; Fang *et al.* 2017), high hydrostatic pressure (Aertsen *et al.* 2005; Fang *et al.* 2017), and NaCl, HCl, EDTA and lactic acid (Łoś *et al.* 2009; Imamovic and Muniesa 2012; Fang *et al.* 2017; Zhang *et al.* 2020).

Bacteriophage induction is an important mechanism by which bacteriophage carry out horizontal transfer of virulence genes between bacteria (Cheetham and Katz 1995). Bacteriophage-mediated *stx*-encoding genes' transfer is not only restricted to intra-species gene exchange among *E. coli* (Brabban *et al.* 2005) as a number of studies have demonstrated the presence of Stx-converting bacteriophages in bacteria other than *E. coli* amongst members of the *Enterobacteriaceae* family (Chen and Novick 2009). Genes coding for Stx have been in the following bacterial species: *Aeromonas* sp, *Citrobacter freundii* (Hayashi et al. 2012), *Enterobacter cloacae* and *E. albertii* (Schmidt *et al.* 1993; Tschäpe *et al.* 1995; Paton and Paton 1996; Strauch *et al.* 2001; Grotiuz *et al.* 2006; Alperi and Figueras 2010; Probert *et al.* 2014). The amino acid sequences and *stx* gene sequences of Shiga toxins expressed by these organisms and STEC strains are homologous, thereby demonstrating evidence of the capacity of *stx*-bacteriophages to transfer and spread through horizontal gene transfer across species. Furthermore, the *stx2* has been found in *Acinetobacter hemolyticus* strain (Grotiuz *et al.* 2006). Clinically, different antimicrobials used in bacterial disease therapy have also been shown to induce *stx2*-encoding bacteriophages and enhance production of Shiga toxin (Yoh *et al.* 1997; Grif *et al.* 1998; Bielaszewska *et al.* 2012). As a result, the use of antimicrobials for STEC disease therapy has become controversial.

While both *stx2 and stx1*-encoding bacteriophages may be induced by DNA-damaging compounds, a few studies have shown that there are differences between *stx1* and

*Stx2*-encoding bacteriophages and their respective toxins (Mühldorfer *et al.* 1996; Wagner *et al.* 2002; Ritchie *et al.* 2003). These studies observed that low iron conditions induced *Stx1* while mitomycin a common recognised prophage inducer could not or hardly induced Stx1 (Mühldorfer *et al.* 1996; Ritchie *et al.* 2003).

#### 2.3.3.1 Induction of *stx*-converting bacteriophages with antimicrobials growth promoters used in food animals

Antimicrobial agents have been used as growth promoters in livestock production systems around the world since the 1950s, administered as in-feed supplements at subtherapeutic concentrations in food animals (Branion and Hill 1951;1952; Branion *et al.* 1953). Eagar et al., reported that 68.5% of antimicrobials (**Table 1**) used as premixes in animal feed comprised primarily macrolides and pleuromutilin's, followed by tetracyclines, sulphonamides, and penicillins (Eagar *et al.* 2012). Additional antimicrobials which are approved for use as growth promoters in food animals (cattle, pigs and poultry) feed in South Africa include **Tetracyclines** (chlortetracycline and oxytetracycline); **Macrolides, lincosamides and pleuromotilins** (tylosin, kitasamycin, josamycin, tilmicosin; tiamulin); **Quinoxalines** (carbadox and olaquindox); **Polypeptides** (bacitracin Zn); **Nitrofurans** (nitrovin); **Ionophores** (monensin, salinomycin, lasalocid); **Streptogramins** (virginiamycin); **Glycopeptides** (flavophospholipol); **Oligosaccharide** (avilamycin); **Phosphonic acids** (Fosfomycin); **Polymeric compounds** (poly 2-propenol 2- propenoic acid) (Eagar *et al.* 2012). All these antimicrobials have been banned in the European Union since 2006 (Barug *et al.* 2006; Castanon 2007; Phillips 2007).

Previous studies showed that subinhibitory concentrations of quinoxalines including olaquindox and carbadox increase *stx2*-converting bacteriophages induction while

tylosin (macrolide) and monensin (ionophore) decrease phage induction and Stx production simultaneously from STEC O157:H7 and *E. coli* C600 (933W) (Kohler *et al.* 2000). Carbadox and olaquindox are approved in South Africa and many countries around the world as additives to pig diets for growth promotion (Eagar *et al.* 2012). Tylosin is a bacteriostatic antimicrobial approved for use in cattle feed to prevent liver abscesses and poultry whereas monensin is authorised as a supplement in cattle, poultry and sheep diets to combat coccidiosis (Dorkov *et al.* 2008; Giguère *et al.* 2013; Cazer *et al.* 2020). In a study by Kim et al, in which subinhibitory concentrations of oxytetracycline and chlortetracycline were tested for induction of stx-converting bacteriophages, it was shown that these antimicrobials could induce and release Stx2 (Kim *et al.* 2016).

### 2.4 Antimicrobial growth promoters used in this study

#### 2.4.1 Virginiamycin

Virginiamycin is a streptogramin antimicrobial compound which was first produced from *Streptomyces virginiae* (Vanderhaeghe *et al.* 1957; Vanderhaeghe and Parmentier 1960; DiCuollo *et al.* 1973; Abou-Youssef *et al.* 1979). Virginiamycin has been used an antimicrobial growth promoter since the 1970s. Virginiamycin is commercialised under two variants virginiamycin M and virginiamycin S which both exhibit bacteriostatic and bactericidal properties separately and in combination, respectively. Virginiamycin acts both gram-positive and a few gram-negative bacteria by inhibits bacterial protein synthesis after binding to the 50 S ribosomal subunit. Virginiamycin is used in cattle, chickens, turkeys and pigs production as an antimicrobial growth promoter to improve nutritional efficiency, increase body weight, and enhance survival of progeny animals and birds (George *et al.* 1982; Singh *et al.*

2008; Stewart *et al.* 2010; Latack *et al.* 2019). Virginiamycin has also been used for nearly 40 years as a prophylactic agent against clostridial enteritis in the U.S.A, Canada and Japan and other countries (George *et al.* 1982; Hao *et al.* 2016). Previously the use of virginiamycin was associated with *Enterococcus faecium* resistance against quinupristin-dalfopristin in food animals which may have led to cross-resistance against pristinamycin and Synercid (Quinupristin-Dalfopristin) and subsequent therapy failure in hospital patients affected with antimicrobial resistant *Enterococcus faecium* infections (Aarestrup *et al.* 2000; Aarestrup *et al.* 2001). Concerns about resistance development in *Enterococcus faecium* in food animals and in hospital patients led to the first ban of virginiamycin use as an antimicrobial promoter in Denmark, in 1999 (Aarestrup *et al.* 1998; Aarestrup *et al.* 2000; Aarestrup *et al.* 2001).

**2.4.2 Flavophospholipol**

Flavophospholipol, also known as flavomycin, bambermycin, or moenomycin A, is a phosphoglycolipid which was produced from a number of *Streptomyce*s strains (Pfaller 2006). It acts on bacterial cells by inactivating peptidoglycan polymerase and impairing transglycolase, thereby preventing cell wall synthesis primarily in Gram-positive bacteria and to a lesser extent in Gram-negative bacteria (Aarestrup *et al.* 1998; Butaye *et al.* 2003). Flavophospholipol is used as an antimicrobial growth promoter in poultry, turkey, cattle and pigs (Crawford 1984; Butaye *et al.* 2003) where it enhances microbiota gut balance as it inhibits growth which creates a barrier against bacterial *Enterococcus faecalis* and *Staphylococcus* spp. This leads to weight gain and improved feed conversion (Crawford 1984; Bolder *et al.* 1999). The use of flavophoslipolipol was associated with reduced *Salmonella* shedding in pigs, calves,

and chicks (Butaye *et al.* 2003). Flavophospholipol is considered an efficient performance enhancer compound which does not kill beneficial intestinal microbiota and can reverse antimicrobial resistance which develops as a result of earlier exposure resistance developed by earlier exposure (Ragland *et al.* 2008). Furthermore, exposure of Gram-positive and Gram-negative bacteria to flavophospholipol does not induce cross resistance or co-select against other antimicrobials (Kissel 1998b;a; Pfaller 2006).

### 2.4.3 Josamycin

Josamycin, or Leucomycin A3, is a macrolide antimicrobial compound, produced by *Streptomyces narbonensis*, subsp. *Josamyceticus* (Strausbaugh *et al.* 1976). It inhibits bacterial protein biosynthesis by reverse binding to the 50S subunit of bacterial ribosome, which inhibits translocation of peptidyl tRNA, causing the polypeptide chains to detach prematurely (Giguère *et al.* 2013). Mainly bacteriostatic, it can also be bactericidal against some bacterial species at higher concentrations. Josamycin is mainly used in pigs and poultry feed to prevent respiratory pathogens *Chlamydia pneumoniae*, *Streptococcus pyogenes*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, *Haemophilus influenzae,* and *Streptococcus pneumoniae*. It also serves as a growth promoting agent in animal husbandry (de la Huebra *et al.* 2007; Giguère *et al.* 2013).

### 2.4.4 Poly 2-propenal 2-propenoic acid

Poly 2-propenal 2 propenoic acid (pPPA) (acrolein) is a polymeric compound consisting of an aldehyde group with antimicrobial activity and carboxylic acid which facilitates water solubility. Poly 2-propenal 2 propenoic acid is a bactericidal

antimicrobial compound which acts on bacteria by cross-linking bacterial lipoproteins on the surface of poultry and weaner pig pathogens (Hampson *et al.* 2000). Poly 2-propenal 2 propenoic acid kills bacterial non-selectively and is active against fungi and viruses (Hampson *et al.* 2000; Murdoch *et al.* 2007). Poly 2-propenal 2 propenoic acid has been shown to be efficient in reducing colonisation of intestinal epithelium with enterotoxigenic enterotoxigenic *E. coli* which causes postweaning diarrhoae in pigs (Hampson 2003). Furthermore, Poly 2-propenal 2 propenoic acid was found to be effective against poultry coccidiosis in a laboratory model (Murdoch *et al.* 2007).

## 2.5 References

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# 3.0 CHAPTER III

## Antimicrobial growth promoters approved in food-producing animals in South Africa induce Shiga toxin-converting bacteriophages from *Escherichia coli* O157:H7.

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### 3.1 Abstract

In this study, four antimicrobial growth promoters, including virginiamycin, josamycin, flavophospholipol, poly 2-propenal 2-propenoic acid and ultraviolet light, were tested for their capacity to induce *stx*-bacteriophages in 47 Shiga toxin-producing *E. coli* O157:H7 isolates. Induced bacteriophages were characterized for Shiga toxin subtypes and structural genes by PCR, DNA restriction fragment length

polymorphisms (RFLP) and morphology features by electron microscopy. Bacteriophages were induced from 72.3% (34/47) of the STEC O157:H7 isolates tested. Bacteriophage induction rates per induction method were as follows: ultraviolet light, 53.2% (25/47); poly 2-propenal 2-propenoic acid, 42.6% (20/47); virginiamycin, 34.0% (16/47); josamycin, 34.0% (16/47); and flavophospholipol, 29.8% (14/47). A total of 98 bacteriophages were isolated, but only 59 were digestible by *Nde*I, revealing 40 RFLP profiles which could be subdivided in 12 phylogenetic subgroups. Among the 98 bacteriophages, *stx2, stx2c* and *stx2d* were present in 85.7%, 94.9% and 36.7% of bacteriophages, respectively. The *Q*, *P*, *CIII*, *N1*, *N2* and IS1203 genes were found in 96.9%, 82.7%, 69.4%, 40.8%, 60.2% and 73.5% of the samples, respectively. Electron microscopy revealed four main representative morphologies which included three bacteriophages which all had long tails but different head morphologies: long hexagonal head, oval/oblong head and oval/circular head, and one bacteriophage with an icosahedral/hexagonal head with a short thick contractile tail. This study demonstrated that virginiamycin, josamycin, flavophospholipol and poly 2-propenal 2-propenoic acid induce genetically and morphologically diverse free *stx*-converting bacteriophages from STEC O157:H7. The possibility that these antimicrobial growth promoters may induce bacteriophages *in vivo* in animals and human hosts is a public health concern. Policies aimed at minimizing or banning the use of antimicrobial growth promoters should be promoted and implemented in countries where these compounds are still in use in animal agriculture.

**Keywords**: antimicrobials, growth promoters, induction, *stx*-converting bacteriophages, STEC O157:H7.

### 3.2 Introduction

Shiga toxin-producing *Escherichia coli* O157:H7 (STEC) causes foodborne disease in humans characterized by watery or bloody diarrhoea. In 5-10% of patients, human STEC disease has been associated with complications including hemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS). Ruminants, including cattle, sheep and goats are the main reservoirs of STEC (Gyles 2007). Humans acquire STEC infections by ingesting STEC-contaminated meat and dairy products, water or vegetables (Hazards 2013; Beutin and Fach 2014; Feng 2014). STEC can be also transmitted from person-to-person and by contact with infected ruminants (Heiman *et al.* 2015).

The major virulence factors of STEC are two antigenically distinct bacteriophages-encoded Shiga toxins: *stx1* and *stx2,* with various genetic variants including four *stx1* (*stx1a*, *stx1c*, *stx1d and stx1e*) and 15 *stx2* (*stx2a, stx2b, stx2c, stx2d, stx2e, stx2f, stx2g, stx2h, stx2i, stx2j, st2k, st2l, stx2m, stx2n and stx2o*) subtypes (Hazards 2013; Gill *et al.* 2022; Lindsey *et al.* 2023). The genetic structure of Shiga toxin-converting bacteriophages (also termed *stx*-converting bacteriophages or *stx*-phages) is similar to that of lambdoid bacteriophages, with immediate early, delayed and late phase transcribed genes (Rodriguez-Rubio *et al.* 2021). Bacteriophage genomes are composed of structural genes which encode proteins responsible for capsid, tail, tail fibers and spike formation. In addition, a number of structural genes encode proteins that regulate virion replication, assembly and release, and Shiga toxin expression. Important structural genes include *Q*, *N* and N2, which encode transcriptional antiterminator and late antiterminator proteins, respectively (Unkmeir and Schmidt 2000; Yokoyama *et al.* 2000). Additional genes located upstream of the *Q* antiterminator include the *cI* repressor and *P,* which are responsible for phage

immunity and DNA replication, respectively (Neely and Friedman 1998; Kruger and Lucchesi 2015).

Previously, it was shown that antimicrobial growth promoters which are supplemented to animal feed at subinhibitory concentrations induce lysogenic *stx*-converting bacteriophages in the STEC chromosome (Kohler *et al.* 2000). Bacteriophage induction occurs when antimicrobials damage DNA and activate the bacterial SOS response, which interferes with virion replication (Little and Mount 1982; Aksenov 1999). At the molecular level, activation of the SOS system leads to derepression of the bacteriophage repressor (CI), which triggers transcription of genes involved in bacteriophage assembly, bacteriolysis and release of free virion particles from STEC (Allison 2007; Smith *et al.* 2007). This phenomenon is considered the main driver of STEC emergence and evolution (Allison 2007; Rodriguez-Rubio *et al.* 2021). Clinically, the use of antimicrobials for treatment of STEC disease in humans has been linked to induction of *stx*-converting bacteriophages with subsequent increase in Stx production and severe STEC disease in humans (Grif *et al.* 1998; Matsushiro *et al.* 1999; Kimmitt *et al.* 2000; Wong *et al.* 2000; Zhang *et al.* 2000) .

Although antimicrobial growth promoters have been banned in the European Union since 2006 (Castanon 2007), these compounds are still used as in-feed additives for livestock growth promotion in many countries around the world, including South Africa. In South Africa, 29% of antimicrobials that are approved for use in livestock growth promotion include compounds banned in the European Union, such as virginiamycin, josamycin, flavophospholipol and poly 2-propenal 2-propenoic acid (Henton *et al.* 2011). Poly 2-propenal 2-propenoic acid cross-links and inactivates surface lipoproteins in the bacterial cell wall leading to bacterial death by lysis (Murdoch *et al.* 2007). Both virginiamycin, a streptogramin, and josamycin, a second-generation

macrolide, inhibit protein synthesis by binding to the 50S subunit of the bacterial ribosome (Butaye *et al.* 2003; Lovmar *et al.* 2009; Li and Seiple 2019). Flavophospholipol is a glycolipid antibiotic which blocks bacterial cell wall synthesis by suppressing peptidoglycan glycosyltransferases (Volke *et al.* 1997).

In this study, the capacity of virginiamycin, josamycin, flavophospholipol, poly 2-propenal 2-propenoic acid, and ultraviolet (UV) light to induce *stx*-converting bacteriophages from STEC O157:H7 was investigated. The four antimicrobial compounds have never been evaluated for their capacity to induce *stx*-converting bacteriophages. Bacteriophage induction assays were conducted *in vitro* on a collection of 47 STEC O157:H7 isolates. Released bacteriophages were further characterized for possession of structural and *stx*-encoding genes, morphology and restriction fragment length polymorphisms (RFLPs).

## 3.3 Results

### 3.3.1 STEC O157:H7 characteristics and bacteriophage induction rates

Bacteriophages were induced from 34/47 (72.3%) STEC O157:H7 isolates tested. The rates of bacteriophage induced per induction method from the 47 STEC O157:H7 isolates were as follows (**Figure 1 and Table 1 and Supplementary Material Table S1**): UV, 53.2% (25/47); poly 2-propenal 2-propenoic acid, 42.6% (20/47); josamycin, 34.0% (16/47); virginiamycin, 34.0% (16/47); and flavophospholipol, 29.8% (14/47). Only 14.9% (7/47) of STEC O157:H7 isolates tested released bacteriophages spontaneously.

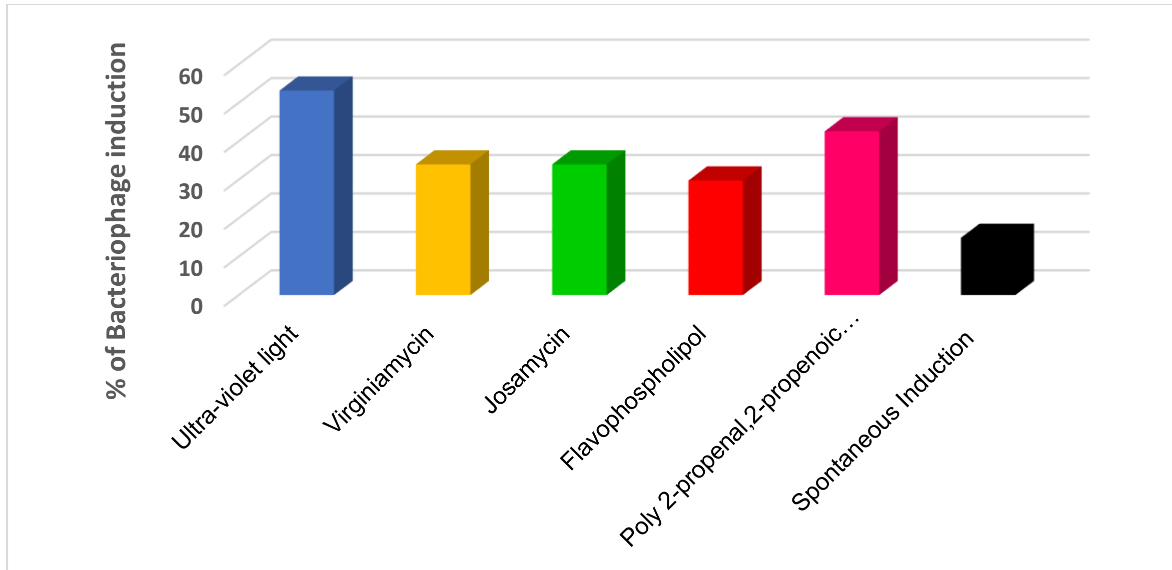



**Figure 1. Stx-converting bacteriophage induction rates by UV and four antimicrobial growth promoters**

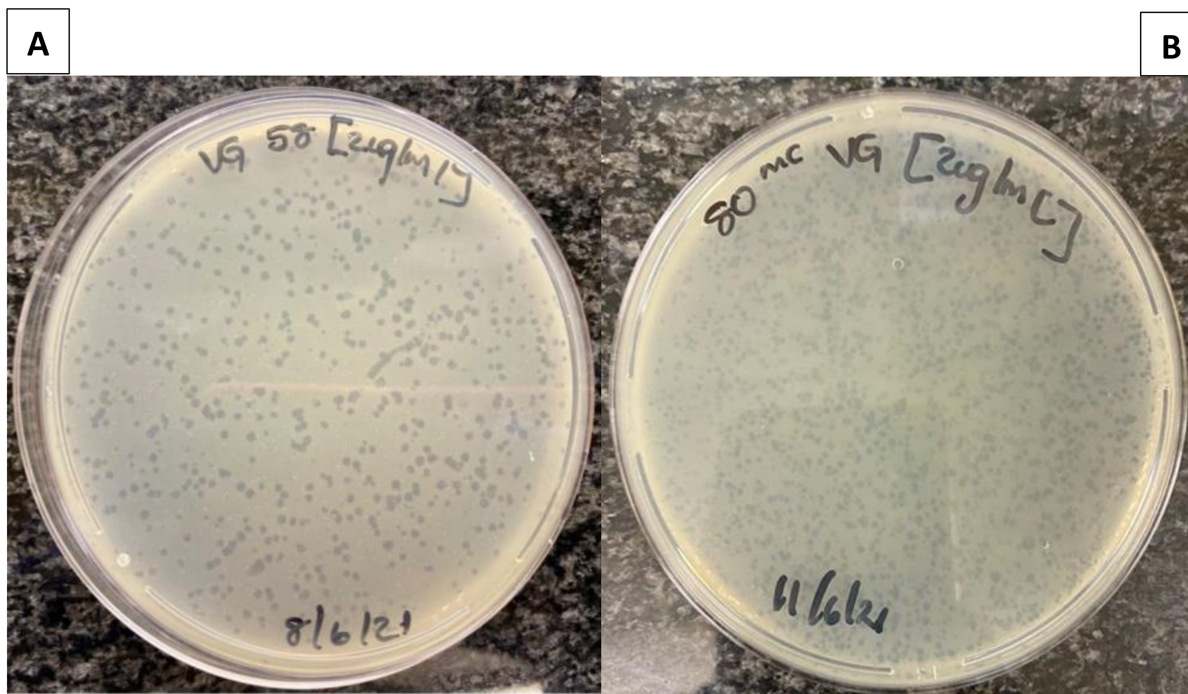



**Figure 2. Bacteriophages plaques on agar plates after induction by virginiamycin.**

**Table 1. Bacteriophages productivity scores per induction method among the 47 STEC O157:H7 isolates.**

| **Bacteriophages infectivity scores** | **Ultraviolet light** | **Virginiamycin** | **Josamycin** | **Flavophospholipol** | **Poly 2-propenal 2-propenoic acid** | **Spontaneous** |
|---|---|---|---|---|---|---|
| 0 (0 plaques) | 4 (16%) | 5 (31.3%) | 2 (12.5%) | 1 (7.1%) | 12 (60%) | 0 |
| 1+ (< 5 plaques) | 2 (8%) | 4 (25%) | 0 | 5 (35.7%) | 1 (5%) | 0 |
| 2+ (5 to 10 plaques) | 15 (60%) | 4 (25%) | 10 (62.5%) | 4 (28.6%) | 2 (10%) | 6 (85.7%) |
| 3+ (> 10 plaques) | 4 (16%) | 3 (18.8%) | 4 (25%) | 4 (28.6%) | 5 (25%) | 1 (14.3%) |
| Total induced bacteriophages | **21/47** | **11/47** | **14/47** | **14/47** | **8/47** | **7/47** |

### 3.3.2 Distribution of bacteriophage-encoded genes.

A total of 98 *stx*-converting bacteriophages were isolated and further genotyped for *stx*-encoding and structural genes **(Supplementary Material Table S1).** PCR genotyping revealed that all induced bacteriophages were *stx2* positive. Overall, the following rates of *stx*-encoding and structural genes were obtained from the 98 induced bacteriophages: *stx2*, 85.7%, (84/98); *stx2c*, 94.9% (93/98); and *stx2d*, 36.7%, (36/98). *P*, 96.9% (95/98); *Q*, 82.7%, (81/98); *CIII,* 69.4, (68/98); *N1*, 40.8 (40/98); *N2*, 60.2, (59/98); *IS1203/*Integrase*,* 73.5 (72/98) (**Supplementary Material-Table S1**).

Bacteriophages productivity scores and presence of *stx*-encoding and structural genes in induced bacteriophages per induction method are depicted in **Figures 3 and 4**, **Table 1** and **Supplementary Materials Table S1.** Among the 25 bacteriophages which were induced by UV, 76% (19/25) were *stx2* positive, 84% (21/25) carried *stx2c*, and 8% (2/25) carried *stx2d*. The following rates were obtained for genes coding for

bacteriophage structural genes: *P*, 96% (24/25); *Q*, 84% (21/25); *CIII*, 48% (12/25); *N1*, 12% (3/25); *N2*, 8% (2/25); and *IS1203* (integrase), 64% (16/25).

Among the 20 bacteriophages induced by poly 2-propenal 2-propenoic, bacteriophage-encoded genes were observed at the following rates: *stx2*, 75% (15/20); *stx2c*, 100% (20/20), *stx2d*, 10% (2/20); *P*, 95% (19/20); *Q*, 65% (13/20); *CIII*, 30% (6/20); *N1*, 45% (9/20); N2, 75% (15/20); *IS1203*, 65% (13/20).

The 16 josamycin-induced bacteriophages revealed the following proportions of genes: *stx2*, 100% (16/16); *stx2c*, 100% (16/16), *stx2d*, 87.5% (14/16); *P*, 93.8% (15/16); *Q*, 81.3% (13/16); *CIII*, 100% (16/16); *N1*, 62.5% (10/16); *N2*, 68.8% (11/16); *IS1203* (integrase), 75% (12/16).

Virginiamycin-induced bacteriophages carried genes at the following rates: *Stx2*, 93.8% (15/16); *stx2c*, 100% (16/16); *stx2d*, 37.5% (6/16); *P*, 100% (16/16); *Q*, 100% (16/16); *CIII*, 93.8% (15/16); *N1*, 43.8% (7/16); *N2*, 81.3% (13/16); and *IS1203* (integrase), 68.8% (11/16). The 14 bacteriophages that were induced by flavophospholipol had the following genes: *stx2*, 85.7% (12/14); *stx2c*, 100% (14/14); *stx2d*, 35.7% (5/14); *P*, 100% (14/14); *Q*, 78.6% (11/14); *CIII*, 92.9% (13/14); *N1*, 35.7% (5/14); *N2*, 92.9% (13/14); and *IS1203* (integrase), 92.9% (13/14). Among the 7 spontaneously induced bacteriophages, the following rates of genes were recorded: *stx2*, 100% (7/7); *stx2c*, 100% (7/7); *stx2d*, 100% (7/7); *P*, 100% (7/7); *Q*, 100% (7/7); *CIII*, 85.7% (6/7); *N1*, 85.7% (6/7); *N2*, 71.4% (5/7); and *IS1203* (integrase), 100% (7/7).

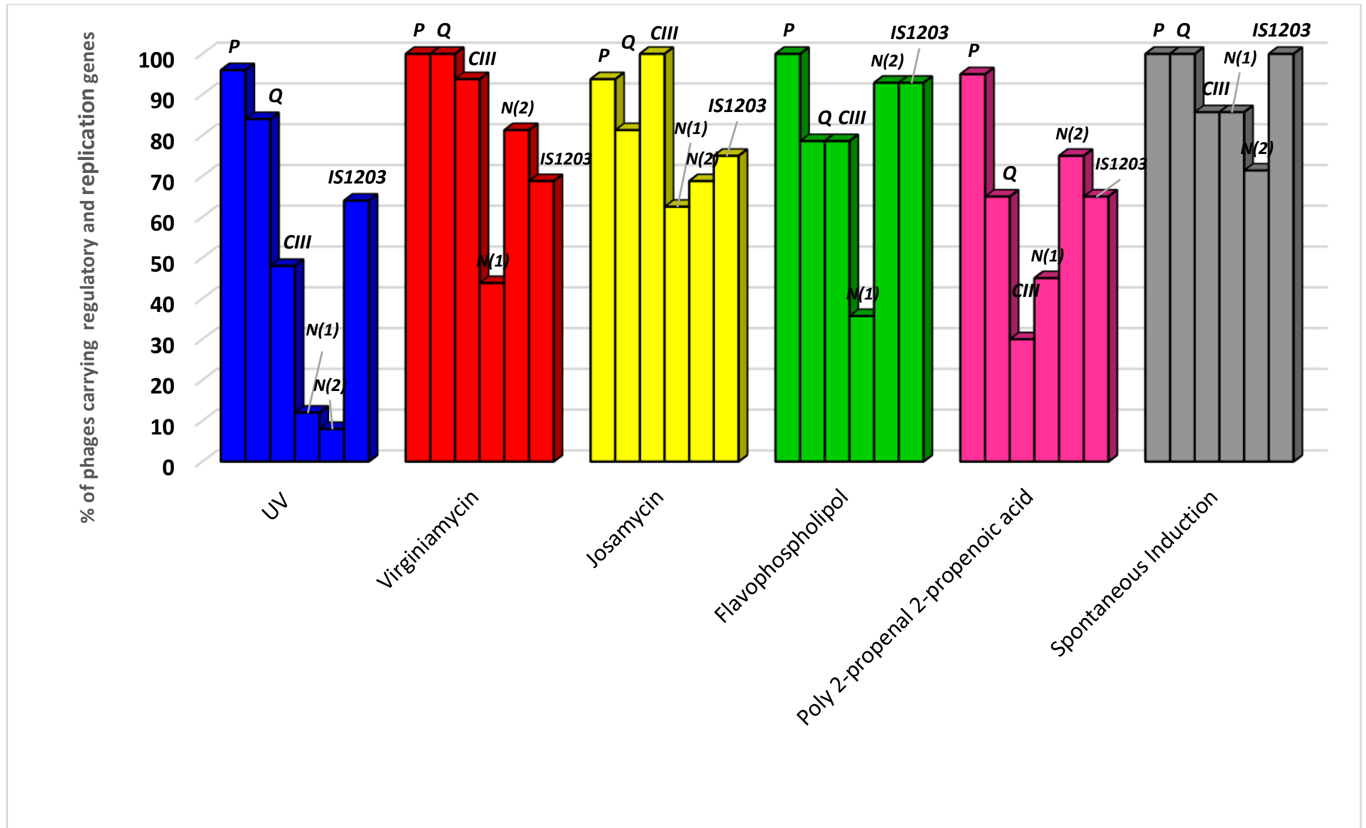



**Figure 3. Distributions of structural genes encoded among on bacteriophages induced with different methods.**

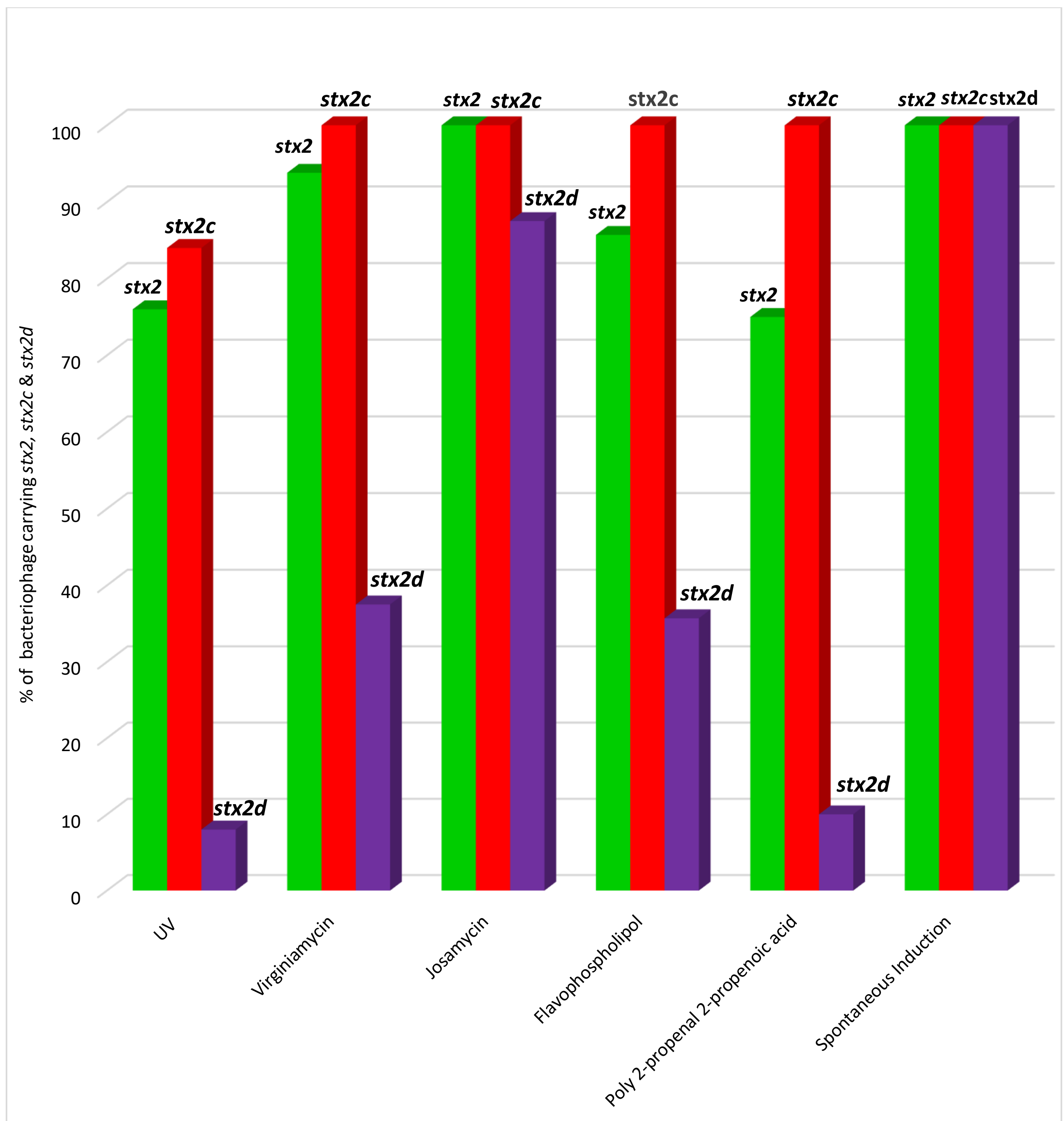



***Figure 4. Distribution of stx2 subtypes among induced bacteriophages.***

### 3.3.3 Restriction fragment length polymorphism of bacteriophage DNA

Bacteriophage DNA was digested using the restriction enzyme *Nde*I (**Figure 6**), and a dendrogram was generated from RFLP digest gel images. Among the 98 induced bacteriophages, only 59 could be digested by *Nde*I. Analysis of the 59 RFLP profiles

displayed 40 bacteriophage subtypes. The 40 subtypes could be assigned to 12 phylogenetic subgroups with a Dice similarity index ≥60% (**Figure 5**). Among the 12 phylogenetic subgroups, three subgroups (9, 10 and 11) were each represented by one bacteriophage. Two subgroups (8 and 12) were represented by two bacteriophages each, three comprised 4 bacteriophages each (3, 6 and 7), one subgroup was represented by nine bacteriophages (subgroup 1), two subgroups comprised 10 bacteriophages each (subgroups 2 and 4) and one (subgroup 5) consisted of 11 bacteriophages.

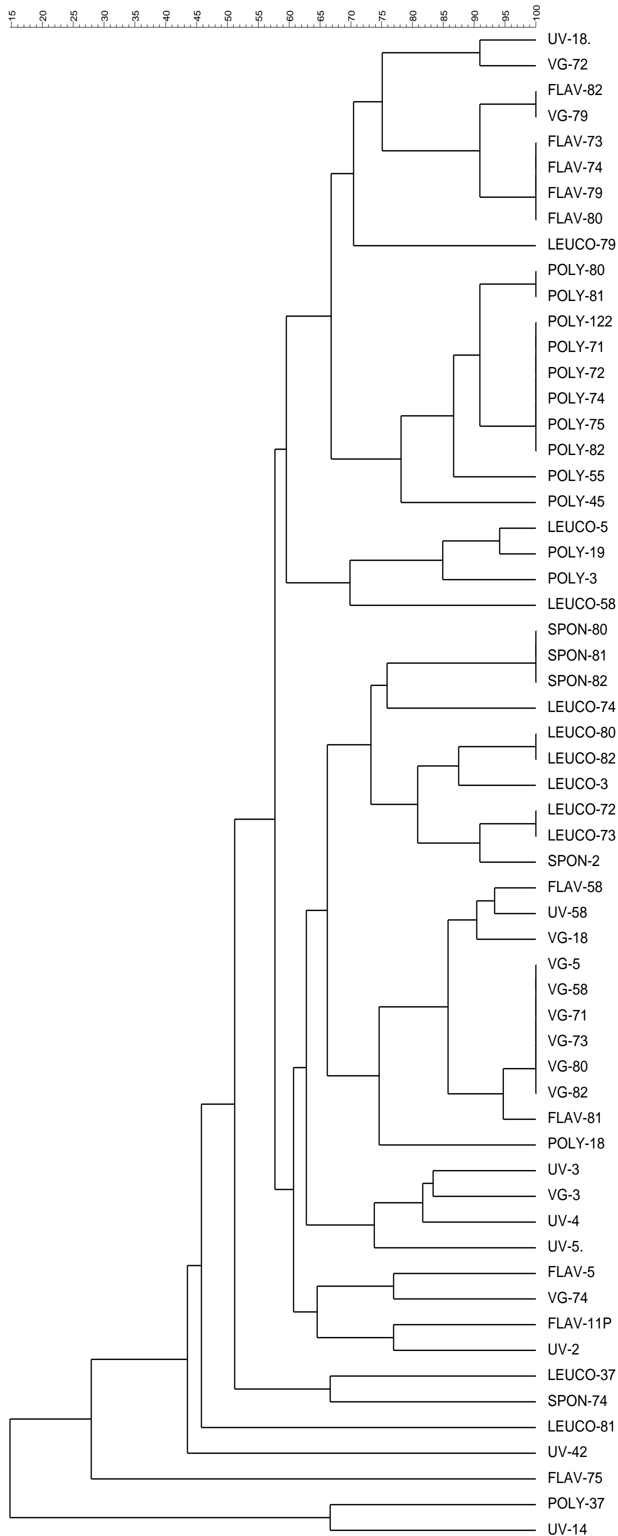



**Figure 5. Dendogram depicting relatedness/diversity among bacteriophages generated from RFLP profiles by *Nde*I digestion.**

c

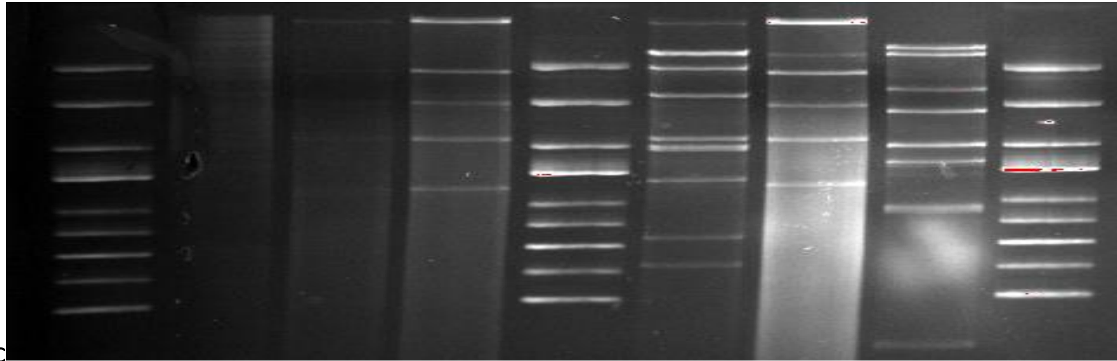
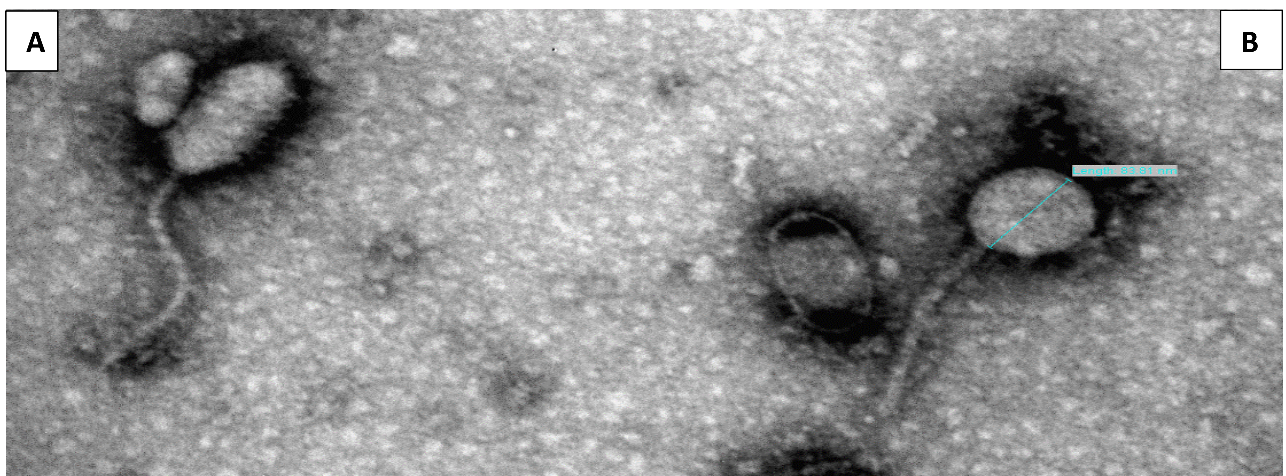


**Figure 6. Electrophoresis in a 0.8% agarose gel digestion by *Nde*I restriction enzyme of Stx bacteriophage DNA induced by flavophospholipol and isolated from cattle O157:H7 strains. M - Molecular size markers. The right side of the figure shows marker band sizes. Bacteriophages 75 and 79 showed very faint bands.**

### 3.3.4. Morphological features and dimensions of bacteriophages

Electron microscopy revealed four morphologies: bacteriophages that possessed elongated icosahedral heads with long tails, oval heads with long tails and hexagonal heads with long tails and hexagonal heads with short thick contractile tail. All these bacteriophages lacked tail fibers (**Figure 7**).



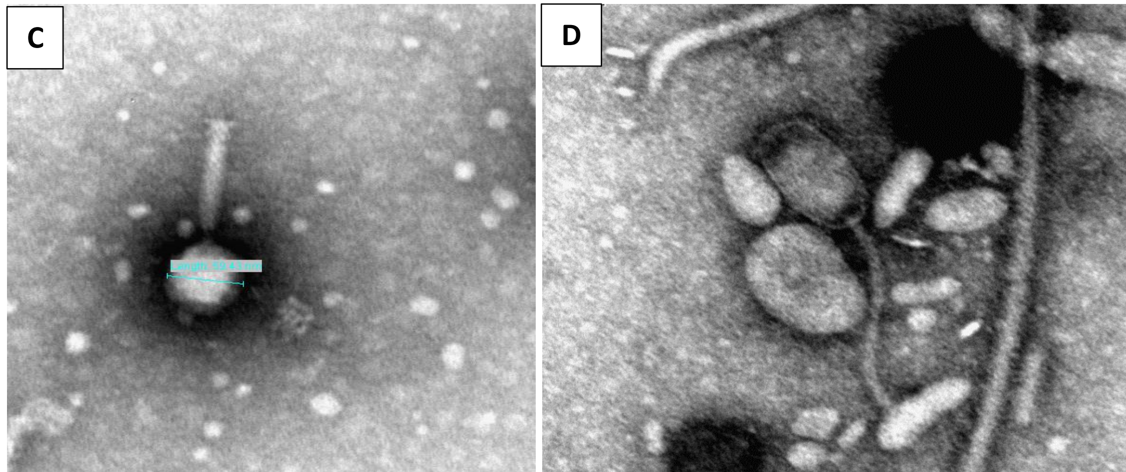



**Figure 7. Electron micrographs of four bacteriophages. (A) Long hexagonal head with a long tail, (B) oval/circular head with a long tail. (C) Icosahedral/hexagonal head with a thick contractile tail. (D) Elongated (oblong) head with long tail. Bars = 200 nm.**

### 3.4 Discussion

In this study, UV and four antimicrobial promoters, including poly 2-propenal 2-propenoic acid, josamycin, virginiamycin and flavophospholipol were tested for their capacity to induce bacteriophages in 47 STEC O157:H7 isolates from humans, cattle and goats. The four antimicrobials are approved for livestock growth promotion in South Africa but have never been tested for their capacity to induce bacteriophages. Overall, it was possible to induce bacteriophages in 72.3% of the STEC O157:H7 isolates using UV light and the four antimicrobial promoters tested including poly 2-propenal 2-propenoic acid, josamycin, virginiamycin and flavophospholipol. As expected, the highest number of bacteriophages (53.2%) was induced by UV irradiation. UV light is considered a standard and effective inducer of *stx*-converting bacteriophages (O'Brien *et al.* 1984; Osawa *et al.* 2000; Allison *et al.* 2003; Karama and Gyles 2008). Zhang et al. (Zhang *et al.* 2020) compared bacteriophage induction by UV irradiation and a number of antimicrobials and showed that UV irradiation induced bacteriophages in the highest number of STEC isolates.

All the four antimicrobial growth promoters which were tested in this study induced plaque-forming *stx*-converting bacteriophages **(Figure 2)**. However, bacteriophage induction rates and productivity scores were highly variable among induced STEC O157:H7 isolates. Poly 2-propenal 2-propenoic acid induced bacteriophages in the highest number of STEC O157:H7 (42.6%), followed by virginiamycin and josamycin (34.0%) and flavophospholipol (29.2%). This finding is consistent with other studies that have also observed variable bacteriophages induction capacity rates among antimicrobials (Grif *et al.* 1998; Kimmitt *et al.* 2000; Kohler *et al.* 2000; McGannon *et al.* 2010; Bielaszewska *et al.* 2012; Carter *et al.* 2021). However, in contrast to previous studies that have shown that *stx*-converting bacteriophages are mainly induced by DNA-damaging antimicrobials that activate the SOS response (Walterspiel *et al.* 1992; Grif *et al.* 1998; McGannon *et al.* 2010; Bielaszewska *et al.* 2012), this study demonstrated that bacteriophages could also be induced by protein (poly 2-propenal 2-propenoic acid, virginiamycin, josamycin) (Murdoch *et al.* 2007; Lovmar *et al.* 2009; Li and Seiple 2019) and peptidoglycan synthesis (flavophospholipol) (Volke *et al.* 1997; Sugimoto *et al.* 2017) inhibiting antimicrobials. Previous studies have shown that antimicrobial agents that block bacterial cell wall or peptiglycan formation or protein synthesis either had no effect, suppressed or decreased *stx*-bacteriophage induction (Yoh *et al.* 1997; Ochoa *et al.* 2007; Pedersen *et al.* 2008; McGannon *et al.* 2010). While it remains unclear why there are differences in bacteriophages induction rates between this study and previous studies which used antimicrobials that inhibit protein and peptidoglycan synthesis capacity in bacterial hosts, variations in bacteriophages induction rates may have been influenced by the use of inadequate or suboptimal antimicrobial subinhibitory concentrations to induce bacteriophages in previous studies (Volke *et al.* 1997; Butaye *et al.* 2003; Lovmar *et al.* 2009; Sugimoto

*et al.* 2017). Furthermore, the observed variations may be due to yet unexplained or unknown factors which are associated with intrinsic characteristics of the STEC O157:H7 isolates induced.

Similarly, as for bacteriophages induction rates, variations in bacteriophages productivity scores were also observed for different antimicrobials used to induce bacteriophages. The majority of isolates induced by josamycin and UV produced the highest number of bacteriophage plaques (more than 10 plaques/isolate). In contrast, the lowest bacteriophage productivity scores (≤5 plaques/isolate) were observed when poly 2-propenal 2-propenoic acid was used to induce bacteriophages, although this compound induced bacteriophages in the highest number of STEC O157:H7 isolates. According to Abedon and Culler, (Abedon and Culler 2007), during bacteriophage induction, high bacteriophages (plaque) productivity scores are a consequence of an optimal and long bacteriophage latent period, while low plaque productivity scores have been associated with a shorter bacteriophage latent period. Once gain, while factors which influence variations in bacteriophage productivity scores cannot be explained, we suggest that bacteriophage productivity scores may also have been positively or negatively influenced by the use of nonptimal subinhibitory concentrations of antimicrobials used to induce bacteriophages.

It is difficult to compare our findings with other studies regarding the capacity of different antimicrobials to induce bacteriophages or influence virion productivity scores because previously similar bacteriophage induction studies were conducted on very small numbers of STEC isolates to allow valid comparisons with the our results (Yoh *et al.* 1997; Yoh *et al.* 1999; Ochoa *et al.* 2007; Pedersen *et al.* 2008; McGannon *et al.* 2010). Moreover, these investigations have yielded mixed results which cannot be compared with this study, depending on whether a direct or indirect method was used

to measure *stx*-bacteriophage induction levels (Yoh *et al.* 1997; Yoh *et al.* 1999; Ochoa *et al.* 2007; Pedersen *et al.* 2008; McGannon *et al.* 2010). In addition, in this study, bacteriophage induction was assessed by the plaque assay technique, which is a direct method for measuring bacteriophage induction and productivity scores. This is unlike previous studies which demonstrated bacteriophage induction using indirect methods by measuring mRNA transcription levels, Stx expression levels or production of free Stx (Kimmitt *et al.* 2000; McGannon *et al.* 2010; Rasko *et al.* 2011). Differences in *stx*-bacteriophages induction rates and productivity scores among the STEC O157:H7 isolates tested may be a reflection of variations in STEC virulence capacity, toxin production levels and disease severity manifestations in human hosts, which can range from mild diarrhoea to severe bloody diarrhoea and complications such as HC and HUS.

Our results also showed that it was possible to induce *stx*-bacteriophages spontaneously in a small number of STEC O157:H7 isolates (14%), consistent with previous studies that have shown that *stx*-bacteriophages can be spontaneously induced from a small number of STEC O157:H7 strains (Bonanno *et al.* 2016; Olavesen *et al.* 2016) under the influence of yet unknown environmental signals, sometimes independent of the RecA-dependent SOS response system (Livny and Friedman 2004). Previous reports have shown that lysogens encoding *stx1* or *stx2* spontaneously released approximately 1 in 20 000-70 000 virion particles per cell generation (Livny and Friedman 2004; Iversen *et al.* 2015). Bullwinkle et al., (Bullwinkle and Koudelka 2011) suggested that spontaneous induction occurs as a result of suboptimal concentrations of repressor needed to activate lytic functions in *stx*-bacteriophages (Bullwinkle and Koudelka 2011; Colon *et al.* 2016). It appears that some *stx*-converting bacteriophages are evolutionarily selected for spontaneous

induction in comparison to bacteriophages which require chemical or physical induction to be induced.

There were 27.7% of STEC O157:H7 isolates which did not induce any bacteriophages. STEC O157:H7 isolates that could not produce bacteriophages may have defective promoters that lack the switch from the lysogenic to the lytic state. Furthermore, bacteriophage induction failure and unsuccessful plaquing capacity may ascribed to expression of colicins that are lethal to *E. coli* strains used for bacteriophage propagation (Allison *et al.* 2003). In addition, some isolates may have not been able to produce bacteriophages because of an unsuitable, immune or insensitive bacteriophage propagation strain.

All 98 induced bacteriophages carried *stx2*, including 75% that possessed *stx2c* while a far lower number (36.7%) possessed *stx2d*. The high frequency of *stx2/stx2c*-encoding bacteriophages and a lower rate of *stx2d*-encoding virion particles was not surprising as these results corresponded to the original characteristics of STEC O157:H7 isolates induced in this study which have been reported elsewhere (Karama *et al.* 2019b; Karama *et al.* 2019a; Malahlela *et al.* 2022). Higher induction rates of *stx2* followed by *stx2c*-encoding bacteriophages and a lower number of *stx2d*-positive virion particles is consistent with previous studies which observed that *stx2* and *stx2c*-encoding bacteriophages were more readily inducible in comparison to *stx2d*-carrying phages (Gobius *et al.* 2003; Muniesa *et al.* 2004; Bonanno *et al.* 2016). Furthermore, Fitzgerald et al., (Fitzgerald *et al.* 2019) reported that *stx2*-encoding bacteriophages were more frequently induced from STEC O157:H7 than *stx2c*. Also, previous reports on the molecular epidemiology of clinical STEC isolates have associated STEC strains that possess *stx2* and *stx2c* with a higher likelihood of severe disease occurrence in humans including HUS (Boerlin *et al.* 1999; Friedrich *et al.* 2002). Moreover, a finding

of lower rates of *stx2d*-encoding bacteriophages was consistent with Gobius et al. (Gobius *et al.* 2003) who observed that *stx2d*-encoding bacteriophages may be noninducible because they are carried on cryptic prophages remnants that lack genes responsible for activation from lysogeny to the lytic replicative cycle.

Out of the 98 induced bacteriophages, only 59 were digestible with the *Nde*I restriction enzyme, while the remaining 39 could not be cut by *NdeI*. Analysis of restriction fragment length polymorphism patterns showed that the 59 bacteriophage restriction profiles belonged to 12 major subgroups, reflecting the diversity (Dice similarity index ≥ 60%) among *stx*-converting STEC O157:H7 bacteriophages. Previous reports have also shown that *stx*-converting bacteriophages are very heterogeneous (Johansen *et al.* 2001; Karama and Gyles 2008; Kruger and Lucchesi 2015; Fagerlund *et al.* 2022). Furthermore, a small portion of bacteriophages which were induced by poly 2-propenal 2-propenoic acid (cluster 2) and leucomycin A3 (cluster 5) induction displayed identical (100%) restriction patterns. There was also a cluster of very closely related bacteriophages which were induced by flavophospholipol (cluster 1). Bacteriophages that clustered together or closely related were released from STEC O157:H7 isolates that originated from the same farm, suggesting the circulation of identical or closely related STEC O157:H7 strains and bacteriophages on farms where these isolates were collected.

Genotyping revealed that the majority of bacteriophages carried the *P* (attachment)*, Q* (antiterminator)*, CIII* (repressor) *N(1)* and *IS1234* (integrase) genes, while the *N(2)* gene, which also codes for integrase was amplified in only 40.8% of bacteriophages, independent of the bacteriophage induction method. This is consistent with previous studies which observed that structural genes are mostly conserved among *stx*-converting bacteriophages of STEC O157:H7 which usually share a common genetic

regulatory system (Unkmeir and Schmidt 2000; Johansen *et al.* 2001; LeJeune *et al.* 2004; Muniesa *et al.* 2004; Ahmad and Zurek 2006). However, while structural genes of *stx*-converting bacteriophages are mostly conserved, variations among these genes have been observed depending on the origin of the STEC O157:H7 isolates harboring them, as previously shown in studies which compared fly with cattle isolates (Ahmad and Zurek 2006) and clinical versus bovine STEC strains (LeJeune *et al.* 2004). The presence of structural genes among *stx*-bacteriophages may also vary depending on the subtype of the gene (Q21 vs *Q33*) (Teel *et al.* 2002) or whether a particular gene subtype is truncated or complete (Llarena *et al.* 2021). Furthermore, Llarena et al., (2021) (Aertsen *et al.* 2005) reported new non-lambdoid *stx*-converting bacteriophages yet undescribed novel sequences of *P* replication initiation genes.

Electron microscopy revealed four main groups of *stx*-converting bacteriophages morphologies including three which all had a long tail with the following representative head shapes: a long hexagonal head, oval/circular head, and elongated (oblong/prolate) head (Muniesa *et al.* 2003; Karama and Gyles 2008; Iguchi *et al.* 2015). A fourth group of bacteriophages had an icosahedral/hexagonal head with a short thick contractile tail. The morphological features of bacteriophages which were observed in this study agreed with previous studies, which found that most *stx*-converting bacteriophages either had elongated or oval heads with long tails or regular hexagonal heads with short tails (Muniesa *et al.* 2003; Karama and Gyles 2008; Iguchi *et al.* 2015).

To our knowledge, this is the first study reporting on the capacity of virginiamycin, josamycin, flavophospholipol and poly 2-propenal 2-propenoic acid to induce bacteriophages from STEC O157:H7 isolates. Our results demonstrated that these four antimicrobials induce *stx*-converting bacteriophages, which are genetically and

morphologically diverse. The induced *stx*-bacteriophages encoded *stx2* and *stx2c* mostly and *stx2d* to a lesser extent. The use of these antimicrobial promoters as in-feed additives in South Africa and other countries around the world may be contributing to STEC emergence and expansion and evolution of new STEC by converting naïve *E. coli* into STEC through lateral gene transfer. This is a public health concern that warrants the formulation of evidence-based policies aimed at stimulating the prudent use of antimicrobials in livestock husbandry. Finding alternative antimicrobial promoters that do not compromise public health or an altogether ban of these compounds in South Africa and other countries where they are still used in animal Agriculture is recommended.

## 3.5 Materials and Methods

### 3.5.1 Bacterial strains

A total of 47 STEC O157:H7 isolates were used for the induction of *stx*-converting bacteriophages. The STEC O157:H7 isolates included 34 cattle (25 beef + 9 dairy), six human and seven goat isolates. Before conducting bacteriophage induction studies, the 47 isolates were reconfirmed as STEC O157:H7 (Paton and Paton 1998; Banjo *et al.* 2018) and screened for *stx1*, *stx2,* two main subtypes including *stx2c* and *stx2d* by PCR (Paton and Paton 1998; Scheutz *et al.* 2012). Briefly, multiplex polymerase chain reaction (mPCR) was performed to detect *stx1* and *stx2* in the STEC isolates using previously described primers and cycling conditions (Paton and Paton 1998). The *stx2* primer (Paton and Paton 1998) is able to detect *stx2d* and most *stx2c* variants as *stx2* (Karama *et al.* 2019b). The *stx2c* variants which cannot be detected by the Paton and Paton (1998) primers as stx2 are usually resolved by the Scheutz *et al*. (2012) primers as *stx2c*. The virulence characteristics of the STEC isolates used

for bacteriophage induction studies have been reported previously (Karama *et al.* 2019b; Karama *et al.* 2019a; Malahlela *et al.* 2022).

**3.5.2 Bacteriophage induction with UV light**

UV light was used as the reference standard bacteriophage induction method against which bacteriophage induction by antimicrobial growth promoters was compared. Bacteriophage induction with UV light irradiation was carried out according to previously published protocols (Karama and Gyles 2008). Briefly, before bacteriophage induction assays, frozen STEC O157:H7 cultures were streaked on Luria Bertani (LB) agar (10 g tryptone, 5 g yeast extract, 10 g/L NaCl) and incubated at 37°C overnight to obtain pure single colonies of STEC O157:H7. A single colony of STEC O157:H7 was added to a 250 ml Erlenmeyer baffled base culture flask (BD Biosciences, Erembodegem, Belgium) containing 45 ml of modified LB broth (10 g tryptone, 5 g yeast extract, 2.5 g/L NaCl and 0.01 M $CaCl_2$). The broth was incubated at 37°C with shaking at 200 rpm for 4 hours to attain exponential growth. After four hours of incubation, the bacterial culture was centrifuged at 4000 rpm for 45 minutes. The pellet was suspended in 5 ml of 0.01 M $CaCl_2$ and transferred onto a glass petri dish. Thereafter, bacteriophage induction was carried out by UV irradiation of the STEC O157:H7 strains according to a previously described protocol (Karama and Gyles 2008). Briefly, the petri dish containing the bacterial suspension was placed inside a biosafety cabinet (Esco AC2-4S1, South Africa) under a UV lamp at 40 cm (Esco, UV-30A, South Africa). The front movable window was covered with aluminum foil to create a dark chamber. Bacteriophage induction was performed by irradiating the bacterial suspension for 120 s with UV light. The UV light wavelength was 254 nm.

The irradiated bacterial suspension was transferred into a sterile 250 mL Erlenmeyer baffled base culture flask (BD Biosciences, Erembodegem, Belgium) containing 45 mL of modified LB broth and incubated at 37°C with shaking (200 rpm) overnight. The overnight culture was centrifuged at 4000 rpm for 45 min, and the supernatant was filtered in a 50 ml centrifuge tube through a 0.45-μm pore-size membrane. Two to three drops of chloroform were added to the filtrate which might be before storage at 4°C, until further processing.

### 3.5.3 Bacteriophage induction with antimicrobial growth promoters

Four antimicrobial growth promoters were tested for their capacity to induce bacteriophages, including josamycin (leucomycin A3), virginiamycin, flavophospholipol and poly 2-propenol 2-propenoic acid (acrolein). All antimicrobials were supplied by Merck (Sigma–Aldrich), South Africa, except for flavophospholipol, which was kindly donated by V-Tech Pty (Ltd), South Africa. Before bacteriophage induction, subinhibitory concentrations (SICs) of virginiamycin, josamycin (leucomycin), flavophospholipol and poly 2-propenal, 2-propenoic acid (acrolein) were determined based on previously published *E. coli* minimum inhibitory concentrations (MICs) (Yamaguchi *et al.* 1978; Jensen *et al.* 2000; Pfaller 2006; Murdoch *et al.* 2007). Bacteriophage induction using antimicrobials was similar to UV induction, with slight modifications. Briefly, STEC O157:H7 isolates were cultured in 45 ml of modified LB broth for 4 h with shaking at 200 rpm at 37°C to attain the exponential growth phase. After 4 h, the exponential growing culture was centrifuged at 4000 X g for 5 min, the supernatant was discarded, and the bacterial pellet was suspended in 5 ml of a 0.01 $CaCl_2$ solution. Bacteriophage induction was performed by adding SICs of antimicrobials to the 5 ml bacterial suspension: virginiamycin (2 μg/mL) (Jensen *et al.*

2000), josamycin **(**128 µg/mL) (Yamaguchi *et al.* 1978) (flavophospholipol (64 µg/mL) (Pfaller 2006) and poly 2-propenal 2-propenoic acid (3 µg/mL) (Murdoch *et al.* 2007). The suspension was added to 45 ml of modified LB and incubated for 16 h-24 h with shaking at 200 rpm at 37°C. After incubation, induced cultures were centrifuged at 4000 X g for 45 min, and the supernatant was filtered through a 0.45-µm pore-size membrane. Two to three drops of chloroform were added to the filtrate, which was stored at 4°C until further processing. Spontaneous bacteriophage induction was also tested by culturing bacteria in modified LB broth for 20 h at 37°C with shaking at 200 rpm followed by centrifugation of the broth at 4000 X g for 45 min followed by filtration of the supernatant through a 0.45-µm pore-size membrane. Two to three drops of chloroform were added to the filtrate which was stored at 4°C, until further processing.

### 3.5.4 Bacteriophage propagation

The double-layer agarose plaque assay technique for isolates and enumeration of phage ʎ was used to propagate bacteriophages and isolate plaques (Sambrook *et al.* 1989). Briefly, 100 µL of the supernatant of the induced culture filtrate was mixed with 100 µL of 0.01 M $CaCl_2$ and 100 µL of an overnight culture of the *E. coli* K-12 MC1061 bacteriophage propagation strain. The mixture was incubated at 37°C for 30 min to allow bacteriophage adsorption, gently mixed with 3 mL soft agarose (100 mL of modified LB broth, 0.5 g agarose) and poured onto a petri dish containing 1.5% modified hard LB agarose (Modified LB, 1.2 g $MgSO_4.7H_2O$, 15 g agarose in 1 liter of $H_2O$). The overlay soft agarose was allowed to solidify and incubated at 37°C overnight while being monitored for plaque formation and productivity for up to 48 h. To rank the different levels of plaque productivity, a scoring system based on the

number of plaque-forming units (PFUs) on agarose plates was applied as follows: +3 > 10 plaques; +2 = 5 to 10 plaques; +1 < 5 plaques; and +0 no plaques.

### 3.5.5 Bacteriophage isolation

Single plaques were harvested from each plate showing plaques by aspirating a single plaque from the surface of the double layer agarose using a sterile glass Pasteur pipette. Aspirated plaques were individually suspended in Eppendorf tubes containing 500 µL of 0.01 M $CaCl_2$ solution. A 100 µL aliquot of the plaque suspension was mixed with 100 µL of 0.01 M $CaCl_2$ and 100 µL of the overnight culture of *E. coli* K-12 strain MC1061 and incubated for 30 min. The solution was gently mixed with 3 mL of soft agar, poured onto hard agar for bacteriophage propagation and incubated overnight at 37°C. Bacteriophages were collected by pouring 5 mL of SM buffer solution (5.8 g NaCl, 2 g $MgSO_4.7H_2O$, 50 mL of 1 M Tris-Cl [pH 7.5], 5 mL of 2% gelatin in 1 liter of molecular grade water) onto petri dishes showing plaques. To obtain an adequate bacteriophage titer for DNA extraction, one plaque was multiplied on five petri dishes. Bacteriophage collection was carried out by pouring SM buffer (100 mM sodium chloride, 10 mM magnesium sulfate, 50 mM Tris-HCl, pH 7.5 and 0.01% (w/v) gelatin) on petri dishes showing plaques. To dislodge bacteriophages from the soft agar, the petri dishes were placed on a platform shaker (FMH Electronics, South Africa) and then incubated with soft shaking for 24-48 h at 4°C. Bacteriophages were harvested by scratching off the top soft agarose from the hard agar and transferring the soft agar/SM buffer suspension mixture to a 50 mL centrifuge tube. Soft agar/SM buffer suspensions from a common single plaque were pooled in a 50 mL tube to obtain an

adequate phage titer and centrifuged at 4000 X g for 30 min. The supernatant was filtered using a 0.45-µm pore-size membrane and transferred into a sterile 50 mL tube. To eliminate any probable residual bacterial contamination, two to three drops of chloroform were added to the bacteriophage filtrate, which was stored at 4°C.

### 3.5.6 Bacteriophage DNA Extraction

DNA was extracted from bacteriophage lysate filtrates using the Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany) supplementary protocol for isolation of single-stranded DNA from M13 phage, according to the manufacturer's instructions. The protocol is based on inactivation of bacterial DNA and RNA in a bacteriophage lysate with DNase and RNase, respectively, followed by precipitation of bacteriophage particles with 30% polyethylene glycol 8000 (Merck, South Africa), lysis of bacteriophages to release DNA, several DNA washings, DNA binding to an anion-exchange resin, DNA elution and precipitation and solution of the precipitated DNA in TE buffer (10 mM Tris, 1 mM EDTA, pH 8).

### 3.5.7 Genetic characterization of induced bacteriophages from STEC O157:H7

PCR was used to screen bacteriophage DNA for genes encoding Shiga toxins (*stx1*, *stx1c*, *stx1d*, *stx2*, *stx2c* and *stx2d*) and bacteriophage structural genes (*P, Q, CIII, N1, N2* and *IS1203*/integrase) (Johansen *et al.* 2001; Latala *et al.* 2001; Smith *et al.* 2007) In addition, to ensure that bacteriophage DNA was not contaminated with STEC bacterial chromosomal DNA, bacteriophage DNA was screened for the chromosomally encoded *eaeA* and *hlyA* genes (Paton and Paton 1998). The PCRs consisted of 25 µL containing 2.5 µL of 10X Thermopol reaction buffer, 2.0 µL of 2.5 mM dNTPs (deoxynucleotide triphosphates), 0.25 µL of 100 mM $MgCl_2$, 0.6 µL of each

primer (10 µM final concentration), 1 U of Taq DNA Polymerase and 5 µL of DNA template. All PCR reagents were procured from New England BioLabs (NEB, Ipswich, MA, USA) except for the primers, which were supplied by Inqaba Biotec (Pretoria, South Africa). DNA from the EDL933 (*E. coli* 0157:H7) strain and sterile molecular grade water were used as positive and negative controls, respectively, in all PCRs.

### 3.5.8 Restriction fragment length polymorphism profiling

RFLP profiling (RFLP) of bacteriophage DNA was carried out by digesting 5 µL of bacteriophage DNA with the *NdeI* restriction enzyme (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's instructions. Bacteriophage DNA was separated by electrophoresis in a 0.8% agarose gel, stained with ethidium bromide (0.5 µg/ml) and visualized with UV light in a Gel Doc system (Bio-Rad, USA). Bionumerics software (Applied Maths, Sint Martens-Latem, Belgium) was used to analyze RFLP patterns and construct dendrograms based on the Dice similarity index (complete linkage, optimization, 1.5%; position tolerance 1.5) and the unweighted pair group method with arithmetic mean (UPGMA).

### 3.5.9 Determination of bacteriophage morphology by electron microscopy

For morphological characterization of bacteriophages, bacteriophages were negatively stained with 3% phosphotungstic acid and examined by electron microscopy (EM). Briefly, a 1 mL suspension of phage supernatant was centrifuged in a Sigma 1-16 ultracentrifuge for 45 minutes, and the pellet was resuspended in **sterile molecular grade** water and a drop of the suspension deposited on a 300-mesh formvar-coated copper grid. The grid was negatively stained with 3% phosphotungstic acid and examined at 80 kV with a Philips CM10 transmission electron microscope at

the EM Unit, Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria).

### 3.6 Author Contributions

Conceptualization, M.K., and N.F.N.N.; data curation, M.K., N.F.N.N. and M.N.M. formal analysis, M.K., and N.F.N.N.; funding acquisition, M.K., E.E., and A.K.; investigation, M.K., M.N.M., and M.C.M.; methodology, M.K, N.F.N.N. and L.G.; resources, M.K., B.T.C.-G. and L.G.; project administration, M.K.; supervision, M.K. and M.C.M.; writing-original draft, M.K. and M.N.M.; writing-review and editing, M.K., N.F.N.N. All authors have read and agreed to the published version of the manuscript.

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### 3.8 Conflict of Interests

None declared.

### 3.9 Ethics statement

The protocol for this study was approved by the Research and Animal Ethics Committees of the Faculty of Veterinary Science, University of Pretoria (REC108-21).

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# 4.0 CHAPTER IV

# GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

## 4.1 General Discussion

Antimicrobials have been used in animal agriculture as in-feed additives to promote livestock growth in various countries worldwide since the 1950s (Branion and Hill 1951;1952; Branion *et al.* 1953). Although antimicrobial growth promoters may be “beneficial” for animal agriculture, these compounds have been found to be detrimental for animal and public health as they have been associated with creating selective pressure which can lead to antimicrobial resistance in bacteria which cause disease in animals and humans (Vidovic and Vidovic 2020; Ma *et al.* 2021). Furthermore, a number of studies have also demonstrated that antimicrobial growth promoters when used at subinhibitory concentrations induce lysogenic *stx*-converting bacteriophages from STEC that are capable of transferring *stx*-encoding genes to naïve *E. coli* (Kohler *et al.* 2000; Bielaszewska *et al.* 2007; Kim *et al.* 2016). As a result of the public health hazards associated with the use of antimicrobial growth promoters, the European Union decided to ban their use in animal agriculture in 2006, making this practice controversial (Barug *et al.* 2006; Phillips 2007).

However, although antimicrobial growth promoters were banned in animal agriculture in Europe since 2006, this controversial practice continues to be implemented in different countries around the world including South Africa. Particularly, a third of antimicrobials which are approved for use as in-feed additive for growth promotion in South Africa include those which were banned in the European Union since 2006 (Henton *et al.* 2011). In this study, four antimicrobial growth promoters approved in South Africa including josamycin, virginiamycin, flavophospholipol and poly 2-propenal

2-propenoic acid were tested for their capacity to induce *stx*-converting bacteriophages in a collection of STEC O157:H7 isolates. Induced bacteriophages were further characterised for possession of structural and *stx*-encoding genes, morphology and restriction fragment length polymorphisms (RFLPs). The four antimicrobials had never been investigated anywhere else for their effect on *stx*-converting bacteriophages induction.

The observations made from this study revealed that josamycin, virginiamycin, flavophospholipol and poly 2-propenal 2-propenoic acid were all capable of inducing *stx*-converting bacteriophages from 73.3% of the tested STEC O157:H7 isolates. Induced bacteriophages mostly carried *stx2* and *stx2c* while a small proportion was *stx2d* positive. The majority of induced bacteriophages possessed structural genes which are typical for lambdoid *stx*-converting bacteriophages. RFLP revealed bacteriophages which were genetically and morphologically diverse. Furthermore, morphological characterisation showed four different representative of bacteriophages groups including three which had 1) elongated icosahedral heads with a long tails, 2) oval heads with long tails and 3) hexagonal heads with long tails, and 4) a fourth group with hexagonal heads and short thick contractile tails.

This study demonstrated that virginiamycin, josamycin, flavophospholipol and poly 2-propenal 2-propenoic acid induced genetically and morphologically diverse *stx*-converting bacteriophages which were mostly *stx2* and *stx2c* but *stx2d* positive to a lesser extent in the majority of STEC O157:H7 isolates tested. While previous studies have used DNA-damaging antimicrobials to induce *stx*-converting bacteriophages in STEC, the particularity of the present study was that the antimicrobials used to induce *stx*-converting bacteriophages were either bacterial petidoglycan or protein synthesis inhibitors. Moreover, this was the first time virginiamycin, josamycin, flavophospholipol

and poly 2-propenal 2-propenoic acid were tested for their bacteriophage induction capacity.

## 4.2 Conclusions

According to our knowledge, this is the first study reporting on the capacity of virginiamycin, josamycin, flavophospholipol and poly 2-propenal 2-propenoic acid to induce bacteriophages from STEC O157:H7 isolates at subinhibitory concentrations *in vitro*. Although these antimicrobial growth promotors may actually enhance animal growth, this study demonstrated that these compounds may also be detrimental for animal agriculture as they may be contributing to emergence of new STEC by inducing bacteriophages which are able to convert naïve *E. coli* into STEC through lateral gene transfer, thus posing a public health concern and threat.

## 4.3 Recommendations

The use of new/alternative antimicrobial growth promoters that do not imperil public health or the complete ban of these compounds in South Africa and other countries where they are still used in animal Agriculture is recommended. In addition, studies on safe alternative compounds that can be used for animal growth promotion are needed. Furthermore, evidence-based policies aimed at promoting the prudent use of antimicrobials in livestock husbandry should be formulated in South Africa and other countries where antimicrobial growth promoters are still used as in-feed additives in animal diets.

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# APPENDIX I

**Supplementary Material. Table S1.** Characteristics of *stx*-converting bacteriophages induced from STEC O157:H7 isolates.

| Phages Induced | Farm | Origin | *NdeI* | Bacteriophage characteristics | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *stx2* | *stx2c* | *stx2d* | *P* | *Q* | *CIII* | *N(1)* | *N(2)* | *IS1203* |
| UV-1 | A | Human | - | + | + | - | + | - | - | - | - | + |
| UV-2 | A | Human | + | + | + | - | + | + | + | - | - | + |
| UV-3 | A | Human | + | + | + | - | + | + | + | - | + | + |
| UV-4 | A | Human | + | + | + | - | + | + | + | - | + | + |
| UV-5 | A | Human | + | + | + | - | + | + | + | - | - | + |
| UV-6 | B | Cattle | - | + | + | - | + | + | + | - | - | + |
| UV-7 | B | Cattle | - | + | + | - | + | + | + | - | - | + |
| UV-8 | C | Cattle | - | - | - | - | + | + | - | - | - | + |
| UV-9 | C | Cattle | - | + | + | - | + | + | - | - | - | + |
| UV-10 | D | Cattle | - | + | + | - | + | + | - | - | - | + |
| UV-11 | C | Cattle | - | - | + | - | + | - | - | - | - | + |
| UV-12 | C | Cattle | - | - | + | - | + | - | - | + | - | + |
| UV-13 | C | Cattle | - | - | + | - | + | + | - | - | - | - |
| UV-14 | C | Cattle | + | + | + | - | + | + | + | - | - | + |
| UV-16 | C | Cattle | - | + | + | - | + | + | - | - | - | - |
| UV-17 | C | Cattle | - | - | + | - | + | + | - | - | - | - |
| UV-18 | C | Cattle | + | + | - | - | + | + | + | - | - | - |
| UV-19 | B | Cattle | - | + | - | - | + | + | - | - | - | - |
| UV-37 | A | Human | - | + | - | - | - | - | - | - | - | - |
| UV-42 | D | Cattle | + | + | + | - | + | + | - | - | - | - |
| UV-58 | C | Cattle | + | - | + | - | + | + | + | - | - | - |
| UV-72 | E | Cattle | - | + | + | + | + | + | + | + | - | + |
| UV-75 | E | Cattle | - | + | - | - | + | + | + | - | - | - |
| UV-81 | E | Cattle | - | + | + | + | + | + | + | + | - | + |
| UV-124 | C | Goat | - | + | + | - | + | + | - | - | - | + |

| Subtotal (%) | | | 8/25 (32) | 19/25 (76) | 20/25 (80) | 2/25 (8) | 24/25 (96) | 21/25 (84) | 12/25 (48) | 3/25 (12) | 2/25 (8) | 16/25 (64) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | ***stx2*** | ***stx2c*** | ***stx2d*** | ***P*** | ***Q*** | ***CIII*** | ***N(1)*** | ***N(2)*** | ***IS1203*** |
| VG-3 | A | Human | + | + | + | + | + | + | + | - | + | - |
| VG-5 | A | Human | + | + | + | - | + | + | + | - | + | + |
| VG-11 | C | Cattle | - | + | + | - | + | + | - | - | - | - |
| VG-14 | C | Cattle | - | + | + | - | + | + | + | - | + | + |
| VG-18 | C | Cattle | + | - | + | - | + | + | + | - | + | - |
| VG-37 | A | Human | - | + | + | - | + | + | + | - | - | - |
| VG-58 | C | Cattle | + | + | + | - | + | + | + | - | + | + |
| VG-71 | E | Cattle | + | + | + | + | + | + | + | - | + | + |
| VG-72 | E | Cattle | + | + | + | + | + | + | + | + | + | + |
| VG-73 | E | Cattle | + | + | + | + | + | + | + | + | + | + |
| VG-74 | E | Cattle | + | + | + | - | + | + | + | + | + | + |
| VG-75 | E | Cattle | - | + | + | - | + | + | + | - | + | - |
| VG-79 | E | Cattle | + | + | + | - | + | + | + | + | + | + |
| VG-80 | E | Cattle | + | + | + | - | + | + | + | + | + | + |
| VG-81 | E | Cattle | - | + | + | + | + | + | + | + | - | + |
| VG-82 | E | Cattle | + | + | + | + | + | + | + | + | + | + |
| **Subtotal (%)** | | | **11/16 (68.8)** | **15/16 (93.8)** | **16/16 (100)** | **6/16 (37.5)** | **16/16 (100)** | **16/16 (100)** | **15/16 (93.8)** | **7/16 (43.8)** | **13/16 (81.3)** | **11/16 (68.8)** |
| | | | | ***stx2*** | ***stx2c*** | ***stx2d*** | ***P*** | ***Q*** | ***CIII*** | ***N(1)*** | ***N(2)*** | ***IS1203*** |
| LEUCO-3 | A | Human | + | + | + | + | + | + | + | + | + | + |
| LEUCO-5 | A | Human | + | + | + | + | + | + | + | - | + | + |
| LEUCO-11 | C | Cattle | - | + | + | - | - | - | + | - | - | - |
| LEUCO-12 | C | Cattle | - | + | + | + | + | - | + | - | - | + |
| LEUCO-14 | C | Cattle | - | + | + | + | + | - | + | - | - | + |
| LEUCO-18 | C | Cattle | - | + | + | + | + | + | + | - | + | - |
| LEUCO-37 | A | Human | + | + | + | + | + | + | + | - | - | - |
| LEUCO-58 | C | Cattle | + | + | + | + | + | + | + | + | + | - |
| LEUCO-72 | E | Cattle | + | + | + | + | + | + | + | + | + | + |
| LEUCO-73 | E | Cattle | + | + | + | + | + | + | + | + | + | + |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LEUCO-74 | E | Cattle | + | + | + | + | + | + | + | + | + | + |
| LEUCO-75 | E | Cattle | - | + | + | + | + | + | + | + | - | + |
| LEUCO-79 | E | Cattle | + | + | + | + | + | + | + | + | + | + |
| LEUCO-80 | E | Cattle | + | + | + | - | + | + | + | + | + | + |
| LEUCO-81 | E | Cattle | + | + | + | + | + | + | + | + | + | + |
| LEUCO-82 | E | Cattle | + | + | + | + | + | + | + | + | + | + |
| **Subtotal**<br>**(%)** | | | **11/16**<br>**(68.8)** | **16/16**<br>**(100)** | **16/16**<br>**(100)** | **14/16**<br>**(87.5)** | **15/16**<br>**(93.8)** | **13/16**<br>**(81.3)** | **16/16**<br>**(100)** | **10/16**<br>**(62.5)** | **11/16**<br>**(68.8)** | **12/16**<br>**(75)** |
| | | | | ***stx2*** | ***stx2c*** | ***stx2d*** | ***P*** | ***Q*** | ***CIII*** | ***N(1)*** | ***N(2)*** | ***IS1203*** |
| FLAV-3 | A | Human | - | + | + | + | + | - | + | - | + | + |
| FLAV-5 | A | Human | + | + | + | + | + | + | + | - | + | + |
| FLAV-11P | C | Cattle | + | + | + | - | + | + | - | - | + | + |
| FLAV-12 | C | Cattle | - | + | + | - | + | - | + | - | + | + |
| FLAV-14 | C | Cattle | - | - | + | - | + | - | + | - | + | - |
| FLAV-58 | C | Cattle | + | - | + | - | + | + | + | - | + | + |
| FLAV-72 | E | Cattle | - | + | + | - | + | + | + | - | + | + |
| FLAV-73 | E | Cattle | + | + | + | + | + | + | + | + | + | + |
| FLAV-74 | E | Cattle | + | + | + | - | + | + | + | + | - | + |
| FLAV-75 | E | Cattle | + | + | + | - | + | + | + | - | + | + |
| FLAV-79 | E | Cattle | + | + | + | + | + | + | + | - | + | + |
| FLAV-80 | E | Cattle | + | + | + | - | + | + | + | + | + | + |
| FLAV-81 | E | Cattle | + | + | + | - | + | + | + | + | + | + |
| FLAV-82 | E | Cattle | + | + | + | + | + | + | + | + | + | + |
| **Subtotal**<br>**(%)** | | | **10/14**<br>**(71.4)** | **12/14**<br>**(85.7)** | **14/14**<br>**(100)** | **5/14**<br>**(35.7)** | **14/14**<br>**(100)** | **11/14**<br>**(78.6)** | **13/14**<br>**(92.9)** | **5/14**<br>**(35.7)** | **13/14**<br>**(92.9)** | **13/14**<br>**(92.9)** |
| | | | | ***stx2*** | ***stx2c*** | ***stx2d*** | ***P*** | ***Q*** | ***CIII*** | ***N(1)*** | ***N(2)*** | ***IS1203*** |
| POLY-3 | A | Human | + | + | + | + | + | + | + | + | + | + |
| POLY-11 | C | Cattle | - | - | + | - | + | - | - | - | - | - |
| POLY-12 | C | Cattle | - | - | + | - | + | - | - | - | - | - |
| POLY-14 | C | Cattle | - | - | + | - | + | - | - | - | - | - |
| POLY-16 | C | Cattle | - | + | + | - | + | + | - | - | + | + |
| POLY-18 | C | Cattle | + | + | + | - | + | + | + | - | + | - |

| POLY-19 | B | Cattle | + | + | + | - | + | + | + | - | + | + |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| POLY-37 | A | Human | + | + | + | - | + | + | + | - | - | + |
| POLY-45 | D | Cattle | + | + | + | + | + | - | - | + | + | + |
| POLY-55 | F | Cattle | + | + | + | - | + | + | + | - | + | + |
| POLY-71 | E | Cattle | + | + | + | - | + | - | - | + | + | + |
| POLY-72 | E | Cattle | + | + | + | - | + | - | - | - | - | - |
| POLY-73 | E | Cattle | - | + | + | - | + | - | - | - | + | - |
| POLY-74 | E | Cattle | + | + | + | - | + | + | + | - | + | + |
| POLY-75 | E | Cattle | + | + | + | - | + | + | - | + | + | + |
| POLY-79 | E | Cattle | - | + | + | - | + | + | - | + | + | + |
| POLY-80 | E | Cattle | + | + | + | - | + | + | - | + | + | + |
| POLY-81 | E | Cattle | + | + | + | - | + | + | - | + | + | + |
| POLY-82 | E | Cattle | + | - | + | - | - | + | - | + | + | + |
| POLY-122 | C | Goat | + | - | + | - | + | + | - | + | + | - |
| **Subtotal (%)** | | | **14/20 (70)** | **15/20 (75)** | **20/20 (100)** | **2/20 (10)** | **19/20 (95)** | **13/20 (65)** | **6/20 (30)** | **9/20 (45)** | **15/20 (75)** | **13/20 (65)** |
| | | | | ***stx2*** | ***stx2c*** | ***stx2d*** | ***P*** | ***Q*** | ***CIII*** | ***N(1)*** | ***N(2)*** | ***IS1203*** |
| SPON-2 | A | Human | + | + | + | + | + | + | - | - | + | + |
| SPON-37 | A | Human | - | + | + | + | + | + | + | + | - | + |
| SPON-74 | E | Cattle | + | + | + | + | + | + | + | + | + | + |
| SPON-75 | E | Cattle | - | + | + | + | + | + | + | + | - | + |
| SPON-80 | E | Cattle | + | + | + | + | + | + | + | + | + | + |
| SPON-81 | E | Cattle | + | + | + | + | + | + | + | + | + | + |
| SPON-82 | E | Cattle | + | + | + | + | + | + | + | + | + | + |
| **Subtotal (%)** | | | **5/7 (71.4)** | **7/7 (100)** | **7/7 (100)** | **7/7 (100)** | **7/7 (100)** | **7/7 (100)** | **6/7 (85.7)** | **6/7 (85.7)** | **5/7 (71.4)** | **7/7 (100)** |
| **TOTAL of POSITIVES** | | | **59/98** | **84/98** | **93/98** | **36/98** | **95/98** | **81/98** | **68/98** | **40/98** | **59/98** | **72/98** |
| **%** | | | **60.2** | **85.7** | **94.9** | **36.7** | **96.9** | **82.7** | **69.4** | **40.8** | **60.2** | **73.5** |
| | | | ***NdeI*** | ***stx2*** | ***stx2c*** | ***stx2d*** | ***P*** | ***Q*** | ***CIII*** | ***N(1)*** | ***N(2)*** | ***IS1203*** |