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The effect of antimicrobials on verocytotoxin bacteriophage transduction under bovine rumen fluid and broth conditions

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Abstract

The verocytotoxin genes in verocytotoxigenic* Escherichia coli *(VTEC) are carried by bacteriophages, incorporated into the bacterial genome (prophage). Antibiotics may promote phage replication and release to infect other cells (transduction), thus leading to the emergence of new VTEC strains. This study investigated transduction of a verocytotoxin2-encoding bacteriophage (3538(vtx_2::cat)) under laboratory conditions, including the effect of antibiotic treatments. Luria-Bertani Miller broth and rumen fluid (raw and sterilised by irradiation) were inoculated with the donor (C600φ3538(Δvtx_2::cat)) and recipient (*E. coli* C600::kanamycin^R) strains (4 $\log_{10}$ cfu/mL) and incubated at 38°C. Antibiotic treatments (minimal inhibitory and sub-inhibitory concentrations of ampicillin, cefquinome, oxytetracycline and sodium sulfamethazine) were applied after 3 h. Samples were tested for donor, recipient, cell-free phage and transductants at times t = 0, 3, 4, 6, 27 (24 h post-antibiotic treatment) and 51 h. Free phage was detected in the untreated broth and rumen samples, as were the transductants confirmed by polymerase chain reaction. The antibiotic treatments did not significantly (P > 0.01) increase the concentrations of free phage or transductants detected. It was therefore concluded that, under laboratory conditions, the antibiotics tested did not induce bacteriophage lysis, release and infection of new bacterial cells beyond that constitutively found in the phage population.

Keywords

antibiotics • rumen • transduction • verocytotoxigenic E. coli *• vtx2 bacteriophage*

Introduction

Escherichia coli are part of the normal intestinal microflora, although some strains cause diarrhoegenic infections in humans. Enteric *E. coli* have traditionally been divided into six pathotypes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), verocytotoxigenic *E. coli* (VTEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (European Food Safety Authority [EFSA], 2013). Two further pathotypes have been proposed by Clements *et al*. (2012), including adherent invasive *E. coli* (AIEC), thought to be associated with Crohn's disease, and the verocytotoxin (Vtx)-producing enteroaggregative *E. coli* (STEAEC) responsible for the 2011 Germany *E. coli* outbreak.

Verocytotoxigenic *E. coli* (VTEC), also referred to as Shiga toxin-producing *E. coli* (STEC), are characterised by the production of Vtxs. These toxins, encoded on bacteriophages, are similar to the toxins produced by *Shigella dysenteriae*. The illnesses associated with VTEC infection range from mild-to-bloody diarrhoea (haemorrhagic colitis) to kidney failure (haemolytic uremic syndrome [HUS]) and thrombocytopenia (Grande *et al*., 2014; Rahal *et al*., 2015).

The most common VTEC serotype associated with human illness worldwide has been *E. coli* O157:H7. This finding may reflect the fact that the majority of infections are caused by this serotype or may be the result of surveillance bias with methods and laboratories focussed on O157:H7. However, in recent years, non-O157 cases have increased and currently outnumber O157 infections in many countries (Monaghan *et al*., 2012). Indeed, the emergence of these new VTEC serogroups has been a cause for serious concern. In 2011, for instance, a major VTEC outbreak occurred, primarily in France and Germany, but also affecting 12 other European Union (EU) countries, the USA and Canada. As on 21 July 2011, there had been 4,321 confirmed cases, of which 852 developed HUS and 54 died (Buchholz *et al*., 2011; Karch *et al*., 2012). The outbreak was unusual as the VTEC strain involved was an EAEC that had acquired a *vtx2* gene (Frank *et al*., 2011).

E. coli acquire the *vtx* genes by phage mediated transduction. When infected by the *vtx*-carrying lambdoid bacteriophage, the phage DNA integrates as a prophage into the bacterial chromosome, where it is replicated and transmitted to daughter cells during each subsequent cell division (James *et al*., 2001;

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Aertsen *et al*., 2005). However, these temperate phages may also replicate lytically. At any given time, a small percentage of the bacteriophage population will be in the lytic phase (Livny and Friedman, 2004; Iversen *et al*., 2015). However, in unfavourable conditions (e.g. antimicrobial treatments), a bacterial regulatory genetic repair mechanism is triggered (also referred to as the SOS response), which is located in a part of the genome that contains the phage genes encoding the lytic phase (Kimmitt *et al*., 2000; Janion, 2001; Schmidt, 2001). Once activated, hundreds of bacteriophages are produced, which are then released into the environment when the bacterial cell lyses (Kohler *et al*., 2000; Colon *et al*., 2016). These phage particles are then free to infect other *E. coli,* resulting in the transfer of the *vtx* genes and the emergence of new VTEC strains (Cornick *et al*., 2006).

Thus, antibiotics that target DNA and/or RNA, such as sodium sulfamethazine (a nucleic acid inhibitor), initiate the lytic cycle (Kohler *et al*., 2000). This antibiotic is commonly used to treat bovine animals for bacterial pneumonia and bovine respiratory disease complex caused by *Pasteurella* spp., colibacillosis (bacterial scours) caused by *E. coli*, acute mastitis *(Streptococcus* spp.) and acute metritis (*Streptococcus* spp.), necrotic pododermatitis (foot rot) (*Fusobacterium necrophorum*) and calf diphtheria (*Fusobacterium necrophorum*). However, other antibiotics, including ampicillin and cefquinome (both of which disrupt cell wall synthesis) and oxytetracycline (which inhibits protein synthesis), are also widely used to treat a range of bovine illnesses but their impact on prophage and horizontal gene transfer by transduction is unknown (Morrison, 1996; Anderson and Yu, 2005; Kim *et al*., 2016). The objectives of this study were therefore to investigate the release of cell free phage and transduction in the following: (1) Luria-Bertani Miller (LB) broth and rumen fluid; (2) LB broth and rumen fluid when sodium sulfamethazine was added at sub- and minimum inhibitory concentrations (SIC and MIC) and (3) LB broth and rumen fluid treated with ampicillin, cefquinome and oxytetracycline (SIC and MIC).

Materials and methods

Bacterial strains

The *E. coli* K-12 strain C600 containing the recombinant vtx_2-encoding lysogenic bacteriophage with a chloramphenicol resistance marker inserted (C600φ3538(Dvtx$_2$::cat)) was used as the donor strain and it was kindly provided by Dr. Herbert Schmidt (Hohenheim University, Germany), while the *E. coli* K-12 strain C600 with kanamycin resistance was used as the recipient strain (C600::kanamycin^R) and was kindly provided by Dr. Maite Muniesa (University of Barcelona, Spain). The *E. coli* K-12 strain C600 without any antibiotic resistance was used to detect φ3538(Dvtx$_2$::cat) plaque formation in the plaque assay procedures. All bacterial cultures were prepared from cryoprotectant beads stored at –20°C and inoculated overnight at 37°C in LB broth (Sigma-Aldrich, Wicklow, Ireland).

Antimicrobial selection and concentration

Commonly prescribed antimicrobials in bovine medicine in Ireland and the wider EU were selected. Each antibiotic was used in the broth and rumen fluid model system at SICs and MICs determined in a preliminary study that demonstrated similar values for the donor (C600φ3538(Dvtx$_2$::cat)) and recipient (C600::kanamycin^R) strains. The antibiotics used inhibited bacterial cell wall or DNA/RNA synthesis, potentially resulting in the bacterial SOS response. The final concentration of the antibiotics used were as follows: sodium sulfamethazine at 100 µg/mL (MIC) and 0.331 µg/mL (SIC); ampicillin at 50 µg/mL (MIC) and 0.011 µg/mL (SIC); cefquinome at 2.5 µg/mL (MIC) and 0.0011 µg/mL (SIC); and oxytetracycline at 25 µg/mL (MIC) and 0.0198 µg/mL (SIC). All antibiotics were obtained from Sigma-Aldrich.

Rumen fluid collection and preparation

One litre of rumen fluid was collected from a ruminally fistulated Friesian cow fed on a grass/grass silage diet on three separate occasions in Teagasc, Moorepark, Co. Cork, Ireland. The rumen fluid was collected by hand and strained through three layers of cheesecloth into a sterile collection bottle placed on ice. Approximately 500 mL of the rumen fluid was left untreated (raw rumen fluid), while 500 mL rumen fluid was irradiated (irradiated rumen fluid) at an ionizing radiation dose of 10 kGy at the Agri-Food and Biosciences Institute in Belfast, Northern Ireland. The irradiated rumen fluid provided a sterile environment by eliminating any background microflora without altering the matrix. Prior to bacterial inoculation, the raw and irradiated rumen fluids were tested for the presence of any background bacteriophages capable of infecting the recipient strain C600, using the method described previously by Nyambe *et al*. (2016). The raw and irradiated rumen fluids were stored at –20°C until required.

Broth and rumen fluid model systems

The broth and rumen fluid model systems were set up according to the Tilley and Terry model (Tilley and Terry, 1963). The broth model system consisted of 50 mL of LB broth placed into 100 mL (20 cm × 3 cm) glass test tubes. The rumen fluid system consisted of 10 mL of raw or irradiated rumen fluid and 40 mL of rumen buffer (26 mM $Na_2HPO_4 \cdot 12H_2O$, 117 mM $NaHCO_3$, 8 mM NaCl, 7.65 mM KCl, 0.63 mM $MgCl_2 \cdot 6H_2O$ and 0.54 mM $CaCl_2 \cdot 6H_2O$) placed into 100 mL glass test tubes (Rivas *et al*., 2010). All test tube samples were flashed with CO_2 for 2 min and immediately sealed with a rubber stopper

fitted with a water-filled gas release valve. Duplicate LB broth, raw rumen fluid and irradiated rumen fluid model systems for every antibiotic treatment and concentration (SIC or MIC) were set up and incubated at 38°C in a water bath for 1 h before inoculation. In addition, LB broth, raw rumen fluid and irradiated rumen fluid model systems without any antibiotic treatment were also set up. After 1 h of incubation, all test tube samples were co-inoculated with 3 mL of C600φ3538(Dvtx$_2$::cat) (donor strain) and 3 mL of C600::kanamycin^R (recipient strain) at a concentration of approximately 4 $\log_{10}$ cfu/mL. The samples were flashed with CO_2 and re-fitted with the rubber stoppers as before (Toomey *et al*., 2009). All test tubes were incubated at 38°C in a water bath without shaking.

Broth and rumen fluid sample analysis

Exactly 1 mL aliquots were taken from each of the untreated and treated LB broth, raw rumen fluid and irradiated rumen fluid test tubes at t = 0 (time of donor and recipient strain co-inoculation), 3 (time of antibiotic addition at SIC or MIC values), 4, 6, 27 (24 h after antibiotic addition) and 51 h (48 h after antibiotic addition). At each time point, 1 mL of the sample was serially diluted in 9 mL of maximum recovery diluent (Sigma-Aldrich) and spread-plated in duplicate. Total viable count (TVC) and total *Enterobacteriaceae* count (TEC) were enumerated only for the untreated and treated raw rumen fluid samples. TVC was enumerated by plating the samples on plate count agar (Sigma-Aldrich) and incubated for 48 h at 30°C, while TEC was enumerated by plating the samples on violet red bile glucose agar (Sigma-Aldrich) and incubated for 24 h at 37°C.

The formation of any putative transductants was analysed by plating on LB agar containing both 30 µg/mL chloramphenicol and 50 µl/mL kanamycin and incubated at 37°C overnight. During the course of the incubation and antibiotic treatment period, the survival of donor strains (C600φ3538(Dvtx$_2$::cat)) was analysed by plating the samples on LB agar containing 30 µg/mL chloramphenicol and that of recipient strains (C600::kanamycin^R) by plating on LB agar containing 50 µl/mL kanamycin and incubating both at 37°C overnight.

Detection of free vtx$_2$ bacteriophage in broth and rumen fluid

To detect the presence of free phage in the untreated and treated LB broth and rumen fluid (raw or irradiated), 2 mL aliquots were extracted at each time point. Each extracted aliquot was centrifuged at 6,797 × *g* for 10 min and filtered using 0.45- µm-sized membrane filters (Sartorius Stedim Ltd., Dublin, Ireland). A serial dilution of the free vtx$_2$ bacteriophage suspension was carried out using LB broth supplemented with a final concentration of 10 mM $CaCl_2$. Exactly 100 µL of the diluted, filtered bacteriophage suspension was added to 100 µL of overnight C600 host cell culture and 125 µL of 0.1 M $CaCl_2$. This mixture was incubated at 37°C for 30 min, after which 3 mL of LB-modified top 0.7% agar (modified by the addition of a final concentration of 10 mM $CaCl_2$ and 0.001% thiamine hydrochloride) was poured onto LB-modified bottom agar (Sigma-Aldrich). The plates were incubated at 37°C overnight and examined for the formation of any 3538(vtx$_2$::cat) bacteriophage plaques.

Confirmation of vtx$_2$ transductants

The DNA of the generated putative transductants obtained on LB agar plates containing both chloramphenicol and kanamycin was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Manchester, UK). Any transductants containing the vtx$_2$ *3538(vtx$_2$::cat)* gene were confirmed by conventional polymerase chain reaction (PCR) using primers HSB1 (5′-CCC GGT ACC ATG AAG TGT ATA TTA TTT AAA TGG-3′) and HSB3 (5′-CCC GCA TGC TCA GTC ATT ATT AAA CTG CAC-3′). The DNA was denatured at 94°C for 10 min (one cycle), followed by 30 cycles of the following protocol: denaturing at 94°C for 30 s, annealing at 53°C for 1 min and extension at 72°C for 1 min; the experiment was concluded with a final extension step at 72°C for 5 min (one cycle) (Schmidt *et al*., 1999).

Volatile fatty acid analysis

Volatile fatty acid (VFA) analysis of the rumen fluid samples was carried out in order to determine the concentration of metabolised nitrogen- and saccharide-based substances. VFA analysis was carried out according to a method described previously by Whelan *et al*. (2012). In brief, approximately 10 mL of bovine rumen fluid (raw or irradiated rumen fluid) was centrifuged at 1,600 × *g* for 15 min. Exactly 200 µL of rumen fluid suspension was added to 3.8 mL distilled water containing 1 mL of internal standard (0.5 g of 3-methylvaleric acid in 1 L of 0.15 M oxalic acid) (Sigma Aldrich). The mixture was centrifuged at 1,600 × *g* for 5 min at 16°C and filtered using 0.45-µm-sized membrane filters (Sartorius Stedim Ireland Ltd., Dublin, Ireland). The resulting filtered supernatant was transferred into 2 mL labelled gas chromatograph vials. VFA concentrations were analysed using a gas chromatograph (Varian 3800 GCL; Varian Inc., Palo Alto, CA, USA) fitted with a 15 m capillary column and coated with 1.20 µm of acid-modified polyethylene glycol (Grace Davison Discovery Sciences, Lancashire, UK).

Statistical analysis

Each experiment was performed in duplicate on three separate occasions, and the mean values were calculated. Data analysis was carried out using analysis of variance (to compare LB broth versus raw rumen fluid versus irradiated rumen fluid and the different antimicrobials) using Genstat version 14.1 (VSN International Ltd., Hemel Hempstead, UK). Significance was determined at $P < 0.05$.

Results

The raw rumen fluid, which had acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acid concentrations of 82.6, 15.7, 2.5, 18.8, 2.9 and 2.4 mmol/L, respectively, tested negative for bacteriophages capable of infecting the host strain (C600) used in these studies. Raw rumen fluid TVC and TEC values ranged from 5.1 to 5.9 $\log_{10}$ cfu/mL and were not affected by sodium sulfamethazine, ampicillin, cefquinome or oxytetracycline, regardless of the concentration applied (SIC or MIC) as their populations increased by approximately 1.0–1.5 $\log_{10}$ cfu/mL after treatment (Table 1). In contrast, their effect on the donor and recipient *E. coli* populations was mixed, depending on the antibiotic, concentration used and the matrix (Tables 2 and 3). At SIC values, a 1–2 $\log_{10}$ cfu/mL decrease in donor *E. coli* populations was obtained in the raw and irradiated rumen fluids when treated with all antibiotics, while recipient concentrations remained relatively stable when antibiotic treatments were applied in LB broth, raw rumen fluid and irradiated rumen fluid. At MIC values in LB broth, both the donor and the recipient *E. coli* C600 populations decreased by up to 5 $\log_{10}$ cfu/mL after ampicillin, cefquinome and oxytetracycline treatments, but sulfamethazine had no effect. A similar pattern was observed in raw rumen fluid and irradiated rumen fluid, although the reductions obtained were generally lower (up to approximately 2 $\log_{10}$ cfu/mL), and sulfamethazine reduced the donor but not the recipient *E. coli* populations (Tables 2 and 3).

Interestingly, cell-free phages were detected in untreated samples of all three matrices at concentrations of up to 2.7 $\log_{10}$ plaque-forming units (pfu)/mL (Tables 2 and 3). There were statistically significant ($P < 0.05$) differences in the cell-free phage concentrations obtained with the different antibiotic treatments depending on the sampling time (0, 3, 4, 6, 27 or 51 h); thus there was no clear pattern wherein one antibiotic consistently achieved significantly ($P < 0.05$) higher cell-free phage concentrations. A range of peak concentrations were obtained in the different matrices: LB broth (2.8 $\log_{10}$ pfu/mL) after 27 h with sodium sulfamethazine (MIC); raw rumen fluid (3.9 $\log_{10}$ pfu/mL) after 4 h with oxytetracycline (SIC); and irradiated rumen fluid (2.7 $\log_{10}$ pfu/mL) after 27 h in untreated samples.

Despite the apparent abundance of cell-free phages obtained in untreated and treated samples, PCR-confirmed transductants were only detected in 18 samples, and the concentrations were low at 0.1–0.8 $\log_{10}$ cfu/mL (Tables 2 and 3). Interestingly, eight positives were obtained in sodium sulfamethazine-treated samples, two with ampicillin, three with cefquinome, three with oxytetracycline and the remainder in untreated samples. The majority were obtained in LB broth (15), with one in raw rumen fluid and three in irradiated rumen fluid.

Discussion

Although it has been suggested that antimicrobials may trigger the proliferation of new phage via the lytic cycle, there is a lack of research demonstrating this process and the potential emergence of new VTEC strains (Kimmitt *et al*., 2000; Free *et al*., 2012; Kim *et al*., 2016). Induction, the process by which phages switch from the lysogenic cycle to the lytic cycle, is triggered by the SOS response in bacteria, which in turn is induced by activation of the RecA protein after binding to single-stranded DNA fragments, which are the products of damaged DNA (Walker, 1984; Sassanfar and Roberts, 1990). DNA damage may be caused by UV radiation, chemicals such as hydrogen peroxide or antibiotics or infection with other phages (Campbell and Reece, 2005; Campoy *et al*., 2006; Keen, 2012). In our study, cell-free phages, at concentrations ranging from 0.4 to 2.7 $\log_{10}$ pfu/mL, were detected in untreated LB broth, raw rumen fluid and irradiated rumen fluid at both SIC and MIC throughout this experiment, suggesting that within a VTEC population, a proportion of the

Table 1. Total viable counts and total *Enterobacteriaceae* counts in untreated and treated raw rumen fluids

Time (h)	TVC, $\log_{10}$ cfu/mL (antibiotics at SIC)					TEC, $\log_{10}$ cfu/mL (antibiotics at SIC)					TVC, $\log_{10}$ cfu/mL (antibiotics at MIC)					TEC, $\log_{10}$ cfu/mL (antibiotics at MIC)				
	NT	Amp	Cef	Sul	Oxy	NT	Amp	Cef	Sul	Oxy	NT	Amp	Cef	Sul	Oxy	NT	Amp	Cef	Sul	Oxy
0	5.8	5.8	5.9	5.6	5.9	5.1	5.5	5.1	5.7	5.8	5.8	5.5	5.2	5.7	5.6	5.1	5.1	5.5	5.1	5.1
3	6.6	6.7	6.6	6.6	7.3	6.0	5.9	5.7	6.3	7.0	6.6	6.8	6.2	6.5	6.9	6.0	6.7	5.9	6.6	5.8
4	6.9	6.9	6.9	6.8	6.7	6.7	6.5	5.8	5.9	6.1	6.9	6.9	6.5	6.2	6.7	6.7	5.6	5.7	4.9	5.9
6	6.8	6.2	6.6	6.5	5.9	6.5	5.7	5.6	6.6	5.9	6.8	6.1	6.5	6.5	6.6	6.5	5.5	5.5	5.9	5.6
27	7.2	6.5	6.5	6.6	6.2	6.7	6.6	6.5	6.5	6.7	7.2	6.1	6.2	6.2	6.5	6.7	6.5	5.8	6.5	6.6
51	6.8	6.6	6.2	6.2	6.6	6.4	6.3	6.2	6.1	6.0	6.8	5.8	6.1	5.9	5.6	6.4	6.3	5.5	6.0	5.0

Amp = ampicillin; Cef = cefquinome; MIC = minimal inhibitory concentration; NT = no treatment; Oxy = oxytetracycline; SIC = sub-inhibitory concentrations; Sul = sodium sulfamethazine; TEC = total *Enterobacteriaceae* count; TVC = total viable count.

phages are constitutively replicating at any given time via the lytic cycle. This finding has been previously reported (Livny and Friedman, 2004; Iversen *et al*., 2015). Such a mechanism would ensure phage persistence even if bacterial inactivation processes were rapid and the VTEC hosts did not survive for sufficient time to allow phage release before being killed (Martinez-Castillo and Muniesa, 2014).

Interestingly, cell-free phage populations peaked after 4–6 h in SIC-treated samples, at 2.0–2.6 $\log_{10}$ pfu/mL, regardless of the medium. Similar peak cell-free phage concentrations were obtained at the MIC values (2.1 $\log_{10}$ $\log_{10}$ pfu/mL in LB broth, 2.4 $\log_{10}$ pfu/mL in raw rumen fluid and 2.7 $\log_{10}$ pfu/mL in irradiated rumen fluid) after the same time period. The decrease in free phages from their peak values was unexpected as viral pathogens are generally considered to survive better that their cellular hosts, a phenomenon that has been demonstrated with Vtx phages (Muniesa *et al*., 1999; Lucena *et al*., 2003; Skraber *et al*., 2004). Moreover, the peak phage concentrations were similar to those previously recorded in human, cattle and poultry waste water and in bovine faeces (Imamovic *et al*., 2010; Rooks *et al*., 2012). As these samples were taken from sources that form part of the natural ecology of *E. coli*, it is unlikely the *E. coli* cells were in an SOS-stressed state, providing further evidence for constitutive replication via the lytic cycle within a Vtx phage population.

Despite cell-free phage being obtained in all three matrices at concentrations of up to 3.9 $\log_{10}$ $\log_{10}$ pfu/mL, transductants were only detected in 20 samples and even then, at low concentrations (0.1–0.8 $\log_{10}$ cfu/mL). A majority of these occurred in LB broth, especially when treated with sodium sulfamethazine. This may reflect the fact that antibiotics that target DNA more effectively induce the lytic phase, even at low concentrations (SIC and MIC) (Kohler *et al*., 2000), but

Table 2. Donor (C600φ3538(Dvtx$_2$::cat)), recipient (*E. coli* C600::kanamycin^R), cell-free phages and PCR-confirmed transductants obtained in untreated and treated (ampicillin: 0.011 µg/mL; cefquinome: 0.0011 µg/mL; sodium sulfamethazine: 0.331 µg/mL; and oxytetracycline: 0.0198 µg/mL) LB broth, raw rumen fluid and irradiated rumen fluid at SIC

Time (h)	Donor: E. coli C600φ3538(Dvtx$_2$::cat), $\log_{10}$ cfu/mL					Recipient: E. coli C600::kanamycinR, $\log_{10}$ cfu/mL					Cell-free phage, $\log_{10}$ pfu/mL					PCR-confirmed transductants, $\log_{10}$ cfu/mL				
	NT	Sul	Amp	Cef	Oxy	NT	Sul	Amp	Cef	Oxy	NT	Sul	Amp	Cef	Oxy	NT	Sul	Amp	Cef	Oxy
LB broth																				
0	4.9^a	4.8^a	5.0^a	4.9^a	4.4^a	4.6^a	4.9^a	5.2^a	4.4^a	5.0^a	0.0^a	0.0^a	0.0^a	0.0^a	0.0^a	ND	ND	ND	ND	ND
3	5.2^a	5.1^a	5.3^a	5.1^a	5.8^a	5.9^a	5.5^a	5.4^a	5.6^a	5.9^a	0.4^a	0.9^a	0.4^a	0.5^a	0.5^a	ND	0.1	ND	ND	0.1
4	6.3^a	6.0^a	4.5^a	5.1^a	4.9^a	6.3^a	6.2^a	5.2^a	5.6^a	5.3^a	1.1^a	1.7^a	0.6^b	2.0^a	2.2^a	0.5	0.5	0.4	0.3	ND
6	6.3^a	5.0^a	4.3^a	5.2^a	4.7^a	6.7^a	5.5^a	5.3^a	5.6^a	4.9^a	2.1^a	0.5^b	1.8^a	1.8^a	1.7^a	ND	0.8	ND	0.2	ND
27	5.9^a	4.6^a	4.5^a	4.6^a	5.4^a	6.5^a	5.3^a	4.9^a	5.6^a	5.3^a	1.0^a	0.8^b	0.7^b	0.7^b	1.8^a	ND	ND	ND	ND	ND
51	4.0^a	4.1^a	3.1^b	4.6^a	4.7^a	5.4^a	4.3^a	5.4^a	5.6^a	4.9^a	0.8^b	0.4^b	1.4^a	0.4^b	0.5^b	ND	0.5	0.3	ND	0.5
Raw rumen fluid																				
0	4.3^a	4.1^a	4.4^a	4.5^a	3.9^a	4.9^a	4.6^a	4.6^a	4.8^a	4.6^a	0.0^a	0.0^a	0.0^a	0.0^a	0.0^a	ND	ND	ND	ND	ND
3	4.5^a	4.5^a	4.5^a	4.2^a	4.3^a	4.8^a	4.9^a	5.5^a	4.9^a	5.0^a	0.5^b	0.5^b	0.5^b	1.1^a	1.2^a	ND	ND	ND	ND	ND
4	4.4^a	4.0^a	4.1^a	4.1^a	3.9^a	5.3^a	4.7^a	4.9^a	5.0^a	4.8^a	0.6^b	2.1^a	2.5^a	2.7^a	3.9^a	ND	ND	ND	ND	ND
6	3.6^b	3.6^b	3.7^b	3.6^b	3.5^b	5.3^a	4.6^a	4.9^a	4.9^a	4.5^a	2.4^a	1.4^b	0.7^b	1.3^b	2.1^a	ND	ND	ND	ND	ND
27	4.2^a	3.7^a	3.3^b	4.4^a	3.9^a	6.0^a	4.6^a	5.0^a	4.4^a	4.7^a	1.4^b	1.0^b	2.0^a	1.1^b	0.7^b	0.8	ND	ND	ND	ND
51	3.8^a	4.0^a	2.2^b	3.0^a	2.2^b	5.6^a	4.4^a	5.0^a	4.5^a	5.0^a	0.7^b	1.8^a	0.7^b	1.3^a	0.6^b	ND	ND	ND	ND	ND
Irradiated rumen fluid																				
0	4.4^a	4.5^a	4.5^a	4.6^a	4.7^a	4.8^a	4.8^a	5.0^a	4.9^a	5.3^a	0.0^a	0.0^a	0.0^a	0.0^a	0.0^a	ND	ND	ND	ND	ND
3	4.8^a	4.7^a	4.7^a	4.6^a	4.8^a	5.6^a	5.5^a	5.5^a	5.4^a	5.2^a	0.5^a	0.4^b	0.4^b	1.0^a	0.5^a	ND	ND	ND	ND	ND
4	4.9^a	4.6^a	4.6^a	5.1^a	4.5^a	6.3^a	5.5^a	4.8^a	4.9^a	5.6^a	2.7^a	0.5^b	1.6^a	1.0^b	0.5^b	ND	ND	ND	ND	ND
6	4.8^a	3.7^b	3.6^b	3.8^b	3.5^b	6.1^a	4.3^a	4.9^a	4.9^a	4.7^a	2.7^a	1.5^a	1.3^b	1.4^b	1.4^b	0.1	ND	ND	ND	ND
27	4.9^a	3.4^b	3.4^b	2.9^b	2.3^c	5.3^a	4.9^a	5.0^a	4.3^a	4.6^a	1.7^a	1.3^b	1.2^b	0.8^b	2.6^a	0.2	ND	ND	ND	ND
51	3.5^a	2.5^b	2.4^b	1.6^b	2.1	5.3^a	4.9^a	4.3^a	4.6^a	4.9^a	0.7^b	2.0^a	1.4^b	0.7^b	1.5^a	ND	ND	ND	0.6	ND

$^{a-c}$Statistical analysis: the same letter indicates statistically similar results ($P < 0.05$) horizontally within a given group (donor, recipient or cell-free phage).

Amp = ampicillin; Cef = cefquinome; LB = Luria-Bertani Miller; NT = no treatment; ND = not detected; Oxy = oxytetracycline; PCR = polymerase chain reaction; SIC = sub-inhibitory concentrations; Sul = sodium sulfamethazine; TEC = total *Enterobacteriaceae* count; TVC = total viable count.

this may also be due to the higher concentration of donor and recipient cells in sodium sulfamethazine-treated samples (Imamovic *et al*., 2009). Indeed, the low overall production and isolation of transductants may be attributed to the combination of donor and recipient cell concentrations being lower than the threshold value required for their generation, due to the decrease in bacterial cell numbers observed in the presence of the different antibiotic treatments (Imamovic *et al*., 2009). Moreover, the free phages observed in our study may have had reduced capacity to infect bacterial cells (Yue *et al*., 2012; Martinez-Castillo and Muniesa, 2014). The higher transduction rates in LB broth may be explained by the impact of the matrix on this process. In general, more complex and heterogeneous matrices, such as rumen fluid, inhibit transduction as the irregular distribution of cells reduces phage–bacteria encounters (Imamovic *et al*., 2009). Based on our findings, the following conclusions were drawn: (1) within a given VTEC population, a proportion of the Vtx phages replicate via the lytic cycle in broth and rumen fluid model systems even in the absence of the bacterial SOS response; (2) sodium sulfamethazine at SIC and MIC promotes this process; and (3) in contrast, other antibiotics, specifically ampicillin, cefquinome and oxytetracycline (at SIC and MIC), have no effect.

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Conflicts of interest

The authors declare no conflicts of interest.

Table 3. Donor (C600φ3538(Dvtx$_2$::cat)), recipient (*E. Coli* C600::kanamycin^R), cell-free phages and PCR-confirmed transductants obtained in untreated and treated (ampicillin: 50 µg/mL; cefquinome: 2.5 µg/mL; sodium sulfamethazine: 100 µg/mL; and oxytetracycline: 25 µg/mL) LB broth, raw rumen fluid and irradiated rumen fluid at MIC

Time (h)	Donor: E. coli C600φ3538(Dvtx$_2$::cat), log$_{10}$ cfu/mL					Recipient: E. coli C600::kanamycinR, log$_{10}$ cfu/mL					Cell-free phage, log$_{10}$ pfu/mL					PCR-confirmed transductants, log$_{10}$ cfu/mL				
	NT	Sul	Amp	Cef	Oxy	NT	Sul	Amp	Cef	Oxy	NT	Sul	Amp	Cef	Oxy	NT	Sul	Amp	Cef	Oxy
LB broth																				
0	4.9^a	4.4^a	4.9^a	4.9^a	4.6^a	4.6^a	4.9^a	4.8^a	5.0^a	4.7^a	0.0^a	0.0^a	0.0^a	0.0^a	0.0^a	ND	ND	ND	ND	ND
3	5.2^a	5.5^a	5.0^a	5.1^a	4.6^a	5.9^a	5.9^a	5.2^a	5.6^a	5.0^a	0.4^a	1.0^a	0.5^a	0.5^a	0.4^a	ND	ND	ND	ND	ND
4	6.3^a	5.6^a	2.8^b	3.4^b	3.6^b	6.3^a	6.3^a	3.0^b	4.0^b	3.8^b	1.1^a	0.6^a	0.5^a	2.3^b	2.1^b	0.5	0.6	ND	ND	ND
6	6.3^a	5.4^a	1.3^b	1.4^b	1.8^b	6.7^a	5.2^a	2.1^b	1.9^b	2.5^b	2.1^a	1.8^a	2.1^a	1.7^a	2.7^a	ND	0.6	ND	ND	ND
27	5.9^a	5.3^a	0.0^b	0.7^b	1.1^b	6.5^a	5.3^a	0.4^b	1.0^b	1.9^b	1.0^a	2.8^b	0.6^a	1.6^a	0.7^a	ND	0.7	ND	ND	ND
51	4.0^a	4.5^a	0.0^b	0.0^b	1.2^b	5.4^a	4.6^a	0.0^b	0.0^b	1.5^b	0.8^a	0.7^a	0.0^a	0.0^a	1.1^a	ND	0.7	ND	ND	0.5
Raw rumen fluid																				
0	4.3^a	4.2^a	4.1^a	4.4^a	3.8^a	4.9^a	4.9^a	4.2^a	4.8^a	4.5^a	0.0^a	0.0^a	0.0^a	0.0^a	0.0^a	ND	ND	ND	ND	ND
3	4.5^a	4.2^a	5.1^a	4.7^a	4.2^a	4.8^a	4.9^a	5.1^a	5.2^a	5.1^a	0.5^b	1.2^b	1.0^b	2.0^a	1.2^b	ND	ND	ND	ND	ND
4	4.4^a	3.7^a	4.2^a	3.7^a	4.4^a	5.3^a	4.9^a	4.8^a	4.8^a	4.7^a	0.6^b	1.2^b	2.7^a	3.0^a	1.3^b	ND	ND	ND	ND	ND
6	3.6^a	3.6^a	2.8^a	2.8^a	3.7^a	5.3^a	4.6^b	4.2^b	4.7^b	4.2^b	2.4^a	2.1^a	1.4^b	1.8^b	1.4^b	ND	ND	ND	ND	ND
27	4.2^a	4.1^a	2.5^b	2.2^b	4.2^a	6.0^a	5.1^b	4.4^b	4.0^b	4.3^b	1.4^b	2.0^a	1.0^b	1.4^b	1.5^b	0.8	ND	ND	ND	ND
51	3.8^a	2.3^b	2.5^b	0.7^c	3.2^a	5.6^a	4.3^a	3.9^a	3.6^a	4.7^a	0.7^b	1.8^a	1.5^a	1.4^a	0.8^b	ND	ND	ND	ND	ND
Irradiated rumen fluid																				
0	4.4^a	4.7^a	4.2^a	4.5^a	4.8^a	4.8^a	4.3^a	4.8^a	4.6^a	4.9^a	0.0^a	0.0^a	0.0^a	0.0^a	0.0^a	ND	ND	ND	ND	ND
3	4.8^a	4.7^a	4.8^a	5.0^a	4.9^a	5.6^a	5.2^a	5.3^a	5.3^a	5.5^a	0.5^b	1.0^a	1.3^a	1.1^a	1.0^a	ND	ND	ND	ND	ND
4	4.9^a	4.9^a	3.9^a	3.6^a	4.1^a	6.3^a	5.6^b	4.7^b	4.9^b	4.9^b	2.7^a	1.0^b	1.2^b	1.2^b	1.2^b	ND	ND	ND	ND	ND
6	4.8^a	4.0^a	2.2^b	2.9^b	3.2^b	6.1^a	4.6^b	3.5^b	3.5^b	4.0^b	2.7^a	2.0^a	1.3^b	1.4^b	1.4^b	0.1	ND	ND	ND	ND
27	4.9^a	3.1^b	1.2^c	1.5^c	3.5^b	5.3^a	4.7^a	2.5^b	3.5^b	3.1^b	1.7^a	2.0^a	2.0^a	1.6^a	2.0^a	0.2	ND	ND	ND	ND
51	3.5^a	2.9^a	0.0^c	0.7^c	2.1^b	5.3^a	5.0^a	2.1^c	3.3^b	3.6^b	0.7^b	1.8^a	1.3^a	1.4^a	1.4^a	ND	ND	ND	ND	ND

$^{a-c}$Statistical analysis: the same letter indicates statistically similar results ($P < 0.05$) horizontally within a given group (donor, recipient or cell-free phage).
Amp = ampicillin; Cef = cefquinome; LB = Luria-Bertani Miller; MIC = minimal inhibitory concentration; NT = no treatment; ND = not detected; Oxy = oxytetracycline; PCR = polymerase chain reaction; Sul = sodium sulfamethazine; TEC = total *Enterobacteriaceae* count; TVC = total viable count.

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