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Original article

Effect of bacteriophage DC22 on *Escherichia coli* O157:H7 in an artificial rumen system (Rusitec) and inoculated sheep

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Abstract — The effect of a bacteriophage, DC22, on the survival of *Escherichia coli* O157:H7 in an artificial rumen system (Rusitec) and in experimentally inoculated sheep was assessed. Plaque assay challenge of *E. coli* strains to DC22 showed that 23 of 23 *E. coli* O157:H7 strains were sensitive to DC22, while 12 non-O157 strains of enterohemorrhagic *E. coli* were not sensitive to the bacteriophage. In triplicate studies using clarified ruminal fluid, the multiplicity of infection (MOI) of DC22 on *E. coli* O157:H7 was determined to be $> 10^4$ plaque forming units (PFU)/CFU. In the artificial rumen system (Rusitec), 10^4 CFU·mL^{-1} of *E. coli* O157:H7 strain 3081 were eliminated from the fermenters (n = 4) 4 h following the administration of 10^5 PFU of DC22/CFU of *E. coli* O157:H7 ($P < 0.05$). *Escherichia coli* O157:H7 persisted in the control fermenters (n = 4) for up to 168 h post-inoculation. Two groups of six wether lambs were fasted for 48 h and orally inoculated with 10^8 CFU of *E. coli* O157:H7 strain E318N. On day 2 post-inoculation, one group was inoculated with 10^5 PFU/CFU of DC22 (DC22-treated group) and the other group was inoculated with an equivalent amount of SM buffer (control group). There was no difference ($P > 0.05$) in the levels of *E. coli* O157:H7 shed by lambs in the DC22-treated group or control group over a 30 day period. Levels of DC22 recovered from the feces of the DC22-treated group declined from 5.98 $\log_{10}$ PFU·g^{-1} on day 3 to undetectable in all lambs on day 13. Non-specific adsorption of DC22 or inactivation prior to reaching the lower tract may have reduced its effectiveness at eliminating *E. coli* O157:H7 from the intestinal tract of lambs.

***E. coli* O157:H7 / bacteriophage / rumen / Rusitec / sheep**

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Résumé — Effet du bactériophage DC22 sur *Escherichia coli* O157:H7 dans un système de rumen artificiel (Rusitec) et de moutons infectés. L'effet du bactériophage DC22, sur la survie d'*Escherichia coli* O157:H7 dans un système de rumen artificiel (Rusitec) et chez des moutons expérimentalement infectés a été évalué. Le test de détection des plages de lyse des souches *E. Coli* au DC22 a montré que 23 des 23 souches *E. Coli* O157:H7 étaient sensibles au DC22, tandis que 12 souches *E. Coli* entérohémorragique non-O157 n'étaient pas sensibles au bactériophage. Sur les études utilisant le fluide ruminal clarifié, la multiplicité d'infection (MI) du DC22 sur *E. coli* O157:H7 a été supérieure à 10^4 unités formant des plages (UFP)/unité formant des colonies (UFC). Dans le rumen artificiel (Rusitec), 10^4 UFC·mL^{-1} de la souche *E. coli* O157:H7 3081 ont été éliminées des fermenteurs (n = 4), 4 h après l'administration du DC22 (MI = 10^5 UFP/UFC, $P < 0{,}05$). En revanche, *E. coli* O157:H7 a persisté dans les fermenteurs témoins (n = 4) jusqu'à 168 h après l'inoculation bactérienne. Après 48 h de jeûne, deux groupes de six agneaux castrés ont été oralement inoculés avec 10^8 UFC de la souche *E. coli* O157:H7 E318N. Deux jours après l'inoculation, un groupe a reçu le DC22 à une MI de 10^5 UPF/UFC (groupe DC22) et l'autre une quantité équivalente de tampon SM (groupe témoin). Aucune différence significative ($P > 0{,}05$) n'a été observée en ce qui concerne les concentrations d'*E. coli* O157:H7 dans les fèces, entre le groupe DC22 et le groupe témoin, sur une période de 30 jours. Les taux de DC22 retrouvés dans les fèces du groupe DC22 ont diminué de 5,98 $\log_{10}$ UFP·g^{-1} le 3^e jour à un niveau indétectable le 13^e jour chez tous les agneaux. L'adsorption non spécifique du DC22 ou son inactivation avant d'atteindre le tractus digestif inférieur a probablement diminué son efficacité à éliminer *E. coli* O157:H7 de la portion intestinale du tube digestif des agneaux.

***E. coli* O157:H7 / bactériophage / rumen / Rusitec / ovin**

1. INTRODUCTION

The human pathogen *Escherichia coli* O157:H7 has become a global public health concern since its initial description in 1982, being implicated in numerous outbreaks of hemorrhagic colitis and the hemolytic uremic syndrome [23, 24]. Although undercooked ground beef and unpasteurized milk have been documented to be the primary vehicles of transmission, foods of non-bovine origin and drinking water have also been linked to *E. coli* O157:H7 outbreaks [4, 15, 33]. Epidemiological investigations, in addition to numerous field surveys, have demonstrated that cattle are a primary reservoir of *E. coli* O157:H7 [15, 17, 35, 37]. Fecal shedding of *E. coli* O157:H7 by cattle is intermittent and seasonal in nature, with peak levels shed during the summer months [5, 18, 22, 35]. Fecal and hide prevalence of *E. coli* O157:H7 has been associated with the contamination of carcasses, suggesting that a role exists for the control of *E. coli* O157:H7 in cattle [12]. Contamination of carcasses during slaughter and processing is likely the primary manner in which *E. coli* O157:H7 is transferred to beef [7, 10, 12].

To date, there is no effective means to control fecal shedding of *E. coli* O157:H7 by cattle. A simulation study conducted by Jordan et al. [19] reported that pre-slaughter intervention strategies, such as the use of agents to reduce the numbers of *E. coli* O157:H7 shed in the feces of cattle, would have the greatest impact on reducing the contamination of carcasses with *E. coli* O157:H7.

Since the discovery of bacteriophages in the early 1900's by Twort and d'Herelle, they have been used successfully to control bacterial pathogens such as *Salmonella*, *Shigella* and *Staphylococcus* [2, 3, 31]. Bacteriophages have also been shown to control enteropathogenic *E. coli* infection in mice [28], calves, piglets and lambs [29, 30]. Bacteriophages specific for *E. coli* O157:H7, isolated from the feces of cattle and sheep, were found to eliminate *E. coli*

O157:H7 in laboratory studies [20]. Recently, Waddell et al. [34] successfully used O157-specific bacteriophages as a means of reducing the duration of *E. coli* O157:H7 fecal shedding in calves. Consequently, bacteriophage therapy may be a natural and effective means of controlling *E. coli* O157:H7 in ruminants.

The objectives of the present study were (i) to assess the effectiveness of a particular bacteriophage, DC22, for its specificity and sensitivity against *E. coli* O157:H7; (ii) to assess the effectiveness of DC22 against *E. coli* O157:H7 in an artificial rumen fermentation system (Rusitec) and (iii) to assess the ability of DC22 to reduce the fecal shedding of *E. coli* O157:H7 in inoculated lambs.

2. MATERIALS AND METHODS

2.1. Specificity and sensitivity of *E. coli* O157:H7 to DC22

Forty bacterial strains (available at Health Canada, Animal Diseases Research Institute, Lethbridge, Alberta) were tested for susceptibility to DC22 (obtained from an anonymous collaborator) (Tab. I). Each of the strains was grown separately in trypticase soy broth (TSB) (BDH, Toronto, ON) for 18 h at 37 °C. The sensitivity and specificity of each strain to DC22 was determined using the plaque titration assay as described by Sambrook et al. [26].

Plate lysate stocks of DC22 were prepared using the soft agar overlay technique and large-scale preparations were prepared from the lysates by liquid infection [26]. A modification of the protocol described by Sambrook et al. [26] was used to concentrate DC22 from the highest titre. Briefly, the lysed culture was centrifuged at 17 700 × *g* for 10 min and a polyethylene glycol 8000 (PEG 8000) (Sigma, St. Louis, MO) solution was added to the supernatant and incubated overnight at 4 °C. The supernatant was centrifuged at 3000 × *g* for 10 min and the PEG 8000 was removed from the white precipitate using 1 M potassium chloride (KCl) (Sigma). The KCl was removed by centrifugation at 12 100 × *g* for 10 min at 4 °C and the supernatant containing the phage was filtered through a 0.45 μm filter followed by 0.20 μm filter.

Bacteriophage DC22 stocks were treated with chloroform, stored at 4 °C and their titre determined immediately prior to use. Luria-Bertani (LB) plates (Difco, Ottawa, ON) prepared to determine whether any bacteria were present in the DC22 stocks were consistently negative. Strains of *E. coli* O157:H7 used for the propagation of DC22 were tested for development of resistance. *Escherichia coli* O157:H7 was separated from 24 h cultures of *E. coli* O157:H7 and DC22 and titred using the plaque titration assay as previously described. The presence and absence of plaques were taken to indicate susceptibility and resistance to DC22, respectively.

2.2. Effect of DC22 on the survival of *E. coli* O157:H7 in the Rusitec

Escherichia coli O157:H7 strain 3081 (kindly made available by W.C. Cray, National Animal Diseases Center, Ames, IA) resistant to 100 $\mu g \cdot mL^{-1}$ ampicillin and 100 $\mu g \cdot mL^{-1}$ kanamycin was used as the bacterial inoculum. Prior to inoculation the *E. coli* O157:H7 strain was grown in TSB for 18 h at 37 °C, centrifuged (4 000 × *g*, 12 min), washed three times in phosphate-buffered saline, pH 7.4 (PBS; 15 mM KH_2PO_4, 8 mM Na_2HPO_4, 137 mM NaCl, 2.6 mM KCl) and re-suspended in PBS. Cells were diluted with PBS to an optical density of 0.5 at 640 nm (~ 10^8 $CFU \cdot mL^{-1}$) (UltraSpec Plus 4054, Pharmacia, Baie d'Urfé, QC) and populations were verified by enumeration on sorbitol MacConkey agar (SMAC; Oxoid, Nepean, ON) supplemented with 2.5 $mg \cdot L^{-1}$ potassium tellurite (Dynal, Lake Success, NY), 0.05 $mg \cdot L^{-1}$

Table I. Plaque assay challenge with bacteriophage DC22.

Bacteria	Plaque formation
E. coli O157:H7	
LRH-69, PT [1] 14, human isolate	+
LRH-70, PT 14, human isolate	+
E32511 (O157:NM), PT 31, human isolate	+
E319, PT 1, human isolate	+
E321, PT 4, human isolate	+
E318N, human isolate	+
HS99-1, PT 14, bovine isolate	+
HS99-2, PT 14, bovine isolate	+
HS99-3, PT 14, bovine isolate	+
HS99-4, PT 14, bovine isolate	+
H4420, PT 87, bovine isolate	+
3081, PT 43, bovine isolate	+
ECI-565, PT 23, bovine isolate	+
ECI-590, PT 49, bovine isolate	+
ECI-596, PT 31, bovine isolate	+
ECI-600, PT 27, bovine isolate	+
ECI-603, PT 49, bovine isolate	+
ECI-605, PT 1, bovine isolate	+
ECI-607, PT 1, bovine isolate	+
ECI-651, PT 32, bovine isolate	+
ECI-652, PT 32, bovine isolate	+
ECI-654, PT Aty[2], bovine isolate	+
ECI-660, PT 8, bovine isolate	+
Enterohemorrhagic *E. coli* (EHEC)	
43426, serotype O103:H25, human isolate	–
9291, serotype O103:H2, human isolate	–
44717, serotype O111:H12, human isolate	–
5529, serotype O103:H4, human isolate	–
52133, serotype O111:K58, human isolate	–
55184, serotype O2:NM, human isolate	–
44131, serotype O26:H11, human isolate	–
33264, serotype O145:H–, human isolate	–
35280, serotype O103:H2, human isolate	–
52050, serotype O111:NM, human isolate	–
5520, serotype O111:K58, human isolate	–
5432, serotype O103:H2, human isolate	–
Other *E. coli*	
EC990984, serotype O55:H7, human isolate (EPEC[3])	+
4582, serotype O26:H11, human isolate (EPEC)	–
1879S1, serotype O157:H7, porcine isolate (VTEC[4])	+
PVT91, serotype O157:KV17:F4, porcine isolate	–
25922 (ATCC[5])	–

[1] Phage type; [2]Atypical phage type; [3]Enteropathogenic *E. coli*; [4]Verocytotoxin-producing *E. coli*; [5]American Type Culture Collection.

cefixime (Dynal), 100 μg·mL^{-1} ampicillin (Sigma) and 100 μg·mL^{-1} kanamycin (Sigma) to yield CT-KASMAC. Plates were incubated for 18–24 h at 37 °C prior to determination of viable numbers.

To determine the optimal multiplicity of infection (MOI) of DC22 for use in the Rusitec, *E. coli* O157:H7 strain 3081 was incubated with DC22 in ruminal fluid in a batch culture incubation. Clarified ruminal fluid (5000 × *g*, 5 min followed by 12 000 × *g* for 30 min) with supplementary glucose (0.2% w/v; Sigma), tryptone (0.1% w/v; Difco) and cysteine-HCl (20 mL·L^{-1} of a 2.5% w/v solution; Sigma) was dispensed to serum vials and equilibrated with CO_2, using oxygen-free CO_2. *Escherichia coli* O157:H7 strain 3081 was added to the serum vials to a final concentration of 10^4 CFU·mL^{-1}. Bacteriophage DC22 was added to the serum vials to achieve desired levels of 10^2, 10^3 and 10^4 PFU/CFU. Phage-free (containing only *E. coli* O157:H7) and cell-free (containing only DC22) serum vials of ruminal fluid were used as controls. Serum vials were incubated at 39 °C and cultured for *E. coli* O157:H7 on CT-KASMAC at 0, 8, 12, 24 and 48 h post-inoculation.

An artificial rumen fermentation system (Rusitec) was used to evaluate the effectiveness of DC22 against *E. coli* O157:H7 under continuous culture conditions. The Rusitec was equipped with eight fermenters (four per treatment, two treatments) each of an 820 mL nominal capacity [11]. Ruminal contents was obtained from two rumen fistulated Hereford heifers fed an 80% barley grain and 20% barley silage diet. Heifers were cared for in accordance with the guidelines of the Canadian Council on Animal Care [9]. Ruminal content was filtered through four layers of cheesecloth to partition it into liquid and solid fractions.

To begin the experiment, each fermenter was filled with 820 mL of filtered ruminal fluid (confirmed *E. coli* O157:H7 negative), pH 6.6. One nylon bag (80 × 160 mm; 51 μm pore size) containing 20 g of solid digesta and one nylon bag containing 10 g of feed (80% barley and 20% silage on a DM basis, ground < 6 mm) were also placed in each fermenter. After 24 h, the nylon bag containing the solid digesta was replaced by a bag containing fresh feed. Thereafter, one feed bag was replaced daily, so that each feed bag remained in the fermenter for 48 h. Fermenters received a continuous infusion of artificial saliva (pH 8.1) at a rate of 0.32 mL·min^{-1} [21]. During set up and during feeding, fermenters were flushed with oxygen-free CO_2. Prior to feeding, pH of the fermenter liquid, vessel volume and production of gas and effluent were monitored daily to ensure that a stable fermentation was achieved. After 8 d of adaptation, an inoculum containing 10^7 CFU of *E. coli* O157:H7 strain 3081 was added to each fermenter in order to obtain a final concentration of 10^4 CFU·mL^{-1} of *E. coli* O157:H7. The two treatments were *E. coli* O157:H7 strain 3081 (control) and *E. coli* O157:H7 strain 3081 + DC22 at a MOI of 10^5 PFU/CFU. Bacteriophage DC22, suspended in SM buffer, was inoculated into the fermenter 8 h after the inoculation of *E. coli* O157:H7 strain 3081. An equivalent amount of SM buffer containing no DC22 was inoculated into the control fermenters. Fermenter liquid samples were collected 30 min post-inoculation, at 4, 8, 12 and 24 h and daily thereafter for enumeration of *E. coli* O157:H7 and DC22.

Escherichia coli O157:H7 strain 3081 was enumerated from ruminal fluid, feed residues and feed bag samples by standard dilution plating onto CT-KASMAC in duplicate. The presence of the O157 antigen was confirmed in 3 sorbitol negative colonies from each plate using *E. coli* O antiserum O157 (Difco). When the organism was no longer detected in the ruminal fluid, feed residues, or feed bags by spread plating, enrichment in modified TSB (mTSB; 20.0 mg·L^{-1} novobiocin (Sigma), 1.5 g·L^{-1} bile salts 3 (Difco), 1.5 g·L^{-1} dipotassium

phosphate (Sigma) and 30 $g \cdot L^{-1}$ TSB) and immunomagnetic separation (IMS) using Dynabeads anti- *E. coli* O157 (Dynal) was performed according to manufacturer's instructions. Enumeration of DC22 from the ruminal fluid was performed using the plaque titration assay [26].

Analysis of variance was performed using the SAS Mixed Model procedure using the spatial model for covariance structure [27]. The repeated measures data were analyzed as a split-plot in time with treatment as the main plot and time as the subplot. The least significant difference (LSD) test was used to determine significant differences ($P < 0.05$) among means.

2.3. Effect of DC22 on the fecal shedding of *E. coli* O157:H7 in lambs

Twelve Romanov wether lambs (4 months of age), divided into 2 groups of 6 animals, were used in a 30 day study. Each group of 6 animals was housed according to the Canadian Council on Animal Care guidelines [9] in a separate climate controlled isolation room containing 2 pens, with 3 lambs in each pen. Lambs were fed a barley-based diet with free access to feed and water throughout the experiment. Animals were adapted to their diets for a 2 week period. On day 20, feed was withdrawn from three animals in each group for 48 h, to assess the effects of feed withdrawal on the fecal shedding of *E. coli* O157:H7 and DC22.

Escherichia coli O157:H7 strain E318N (kindly made available by A. Borczyk, Enteric Reference Laboratory, Ministry of Health, Toronto, ON) resistant to 40 $\mu g \cdot mL^{-1}$ nalidixic acid was used as the bacterial inoculum for the lambs. The *E. coli* O157:H7 inoculum was prepared as previously described and viable numbers were confirmed by standard dilution plating on SMAC supplemented with 2.5 $mg \cdot L^{-1}$ potassium tellurite, 0.05 $mg \cdot L^{-1}$ cefixime and 40 $\mu g \cdot mL^{-1}$ nalidixic acid (Sigma) to give CT-SMACnal. Each lamb was orally inoculated with 50 mL of PBS containing 10^8 CFU *E. coli* O157:H7 strain E318N using a sterile 60-mL syringe (Fisher, Nepean, ON) and stomach tube. On day 2 post-inoculation, the 6 lambs in the DC22-treated group were inoculated with 10^{13} PFU of DC22 suspended in SM buffer, using a sterile 60-mL syringe and stomach tube. The 6 control lambs were orally inoculated with an equivalent amount of SM buffer without DC22.

Fecal samples collected from the lambs by rectal palpation were placed in sterile polypropylene specimen containers (Fisher) and transported to the laboratory for analysis within 1 h. Fecal samples were taken at the same time daily for 8 d post-inoculation, followed twice weekly thereafter for a total time period of 30 d. The absence of *E. coli* O157:H7 prior to inoculation was confirmed by collecting fecal samples which were cultured using enrichment in mTSB followed by IMS using Dynabeads anti- *E. coli* O157, and plating onto SMAC supplemented with 2.5 $mg \cdot L^{-1}$ potassium tellurite and 0.05 $mg \cdot L^{-1}$ cefixime (CT-SMAC) and CT-SMACnal.

Escherichia coli O157:H7 strain E318N was enumerated from fecal samples by standard dilution plating onto CT-SMACnal in duplicate. Plates were incubated for 18–24 h at 37 ^{o}C prior to determination of viable numbers. The presence of the O157 antigen was confirmed in 3 sorbitol negative colonies from each plate using *E. coli* O antiserum O157. Multiplex PCR (O157 specific) assays as described by Gannon et al. [13] were used as further verification on one fecal sample a week in which the experimental strain was recovered (*E. coli* O157:H7 strain E318N). Enrichment and IMS using Dynabeads anti- *E. coli* O157 was performed on fecal samples when *E. coli* O157:H7 strain E318N was no longer detected by standard dilution plating.

The titre of DC22 in the fecal samples of the lambs in the treatment and control groups was determined using the plaque titration assay, commencing on day 3 and on each sampling day thereafter [26]. An aliquot (1 mL) of the 1:10 fecal dilution in PBS was centrifuged at 10 000 $\times g$ for 10 min in order to sediment the fecal material. The supernatant was then filtered using a 0.20 μm syringe filter (Fisher) and titred for DC22 using *E. coli* O157:H7 strain E318N. Fecal samples were tested for the presence of *E. coli* O157-specific bacteriophages prior to inoculation of the lambs with DC22.

Analysis of variance was performed using the SAS Mixed Model procedure using the spatial model for the covariance structure [27]. The repeated measures data were analyzed as a split-plot in time with diet as the main plot and time as the subplot. The least significant difference (LSD) test was used to determine the differences among means where significant effects were observed ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Phage specificity and sensitivity of *E. coli* O157:H7 to DC22

The bacteriophage DC22 produced plaques on all the *E. coli* O157:H7 strains tested (Tab. I). Plaques manifested on all strains were approximately 0.90 mm in diameter. In order to determine specificity for *E. coli* O157:H7, DC22 was also tested against other enterohemorrhagic *E. coli* (EHEC). None of the non-O157 EHEC tested produced plaques. Activity of DC22 was observed against O55:H7 strain EC990984 (enteropathogenic *E. coli*, EPEC) and O157:H7 strain 1879S (Verocytotoxin-producing *E. coli*, VTEC), but not against *E. coli* O157:KV17:F4 strain PVT91 (porcine isolate).

The adsorption of phages to bacterial cells is the first step of infection and is dependent on the presence of highly specific receptors on the bacterial cell wall to which the phage tail fibers bind [1, 16]. Our results indicate that while DC22 appears to be specific for *E. coli* O157:H7 and closely related strains, the phage receptor may not be the O157-antigen since *E. coli* O157:KV17:F4 strain PVT91 was not lysed by DC22. In contrast, the EPEC strain O55:H7, a putative progenitor of *E. coli* O157:H7, was lysed by DC22. This suggests that the receptor for DC22 is a cell wall constituent which is common to *E. coli* O157:H7 and *E. coli* O55:H7.

Kudva et al. [20] reported that phage infection was influenced by the nature of the host cell O157 lipopolysaccharide (LPS) in three O157-specific phages isolated from ovine and bovine fecal samples. It has been reported that phage attachment sites on bacterial cells do not necessarily correspond to the antigenic sites on the cell [25]. Surface exposed LPS and the porin protein OmpF, have been shown to serve as phage receptors in *E. coli* K-12 [32]. A cooperative interaction between OmpC and LPS was required for the efficient binding of phage AR1, which specifically infects *E. coli* O157:H7 [25, 36]. Further studies are required in order to establish the specific receptor for DC22 in *E. coli* O157:H7 strains.

3.2. Effect of DC22 on the survival of *E. coli* O157:H7 in the Rusitec

The MOI of DC22 necessary for lysis of *E. coli* O157:H7 was higher than anticipated. At levels of 10^2 and 10^3 PFU/CFU, DC22 was not effective in significantly reducing levels of *E. coli* O157:H7 in clarified ruminal fluid. An MOI of 10^4 CFU/PFU DC22 resulted in a decrease in levels of *E. coli* O157:H7 strain 3081 from 3.66 to 1.43 $\log_{10}$ CFU·mL^{-1} over the 48 h incubation period (Fig. 1). However, since 10^4 PFU/CFU did not result in complete inhibition of

E. coli O157:H7 an MOI of 10^5 PFU/CFU was used in subsequent tests in the Rusitec.

The phage DC22 at an MOI of 10^5 PFU/CFU was successful in eliminating *E. coli* O157:H7 strain 3081 from ruminal fluid within 4 h of its inoculation into the fermenters. In the control fermenters, numbers of *E. coli* O157:H7 declined steadily until 120 h post-inoculation, at which time enrichment and IMS was required in order to detect the organism. The inoculated strain was detected in the control fermenters up to 168 h after inoculation (Fig. 2A).

The decline in numbers of DC22 (Fig. 2B) indicates that the phage did not replicate and form infective particles in *E. coli* O157:H7 in the ruminal fluid. It is possible that the high MOI resulted in premature lysis of *E. coli* O157:H7 without phage progeny being liberated. This phenomenon has been reported to occur when bacteria are attacked by a very large number of phage particles. Lysis of the bacteria is due to damage to the host cell membrane by the phage particles rather than to infection in the usual manner [1].

Experiments using clarified ruminal fluid indicated that while a 10^4 PFU/CFU MOI resulted in a reduction in levels of *E. coli* O157:H7 (Fig. 1), re-growth of the uninhibited organisms may result in a subsequent increase in the levels of *E. coli* O157:H7. This is in agreement with a study conducted by Kudva et al. [20] who found that no single phage was able to successfully eliminate *E. coli* O157:H7 from cultures *in vitro*. A mixture of three O157-specific phages was necessary to eliminate *E. coli* O157:H7 from cultures, with aeration, an incubation temperature of 37 °C and a high MOI being critical for rapid cell lysis [20]. While aeration is important in increasing the opportunity for phage-bacterium interaction and cell infection, it appears that the mixing which occurred in the fermenters of the Rusitec was sufficient to allow for adsorption of the phage particles to the bacteria. Furthermore, the population of *E. coli* O157:H7 was naturally declining as a result of the failure of *E. coli* O157:H7 to establish under the continuous fermentation systems within the Rusitec. Although *E. coli* O157:H7 has been isolated from the rumen [6] it is thought to be transient within this environment with resident populations being established primarily in the cecum and colon [8, 14].

There was a steady decrease in levels of DC22 recovered from the ruminal fluid in the DC22– treated fermenters over the 192 h experimental period (Fig. 2B). The infusion of artificial saliva (pH 8.1) at a rate of 0.32 mL·min^{-1} served to buffer the bacterial fermentations within the fermentation vessels to a pH of between 6 and 6.5, simulating rumen conditions in vivo. A one $\log_{10}$ reduction in levels of DC22 in the fermenters was observed 48 h post-inoculation, while an additional one $\log_{10}$ decrease was recorded after 120 h with a 3.0 $\log_{10}$ reduction occurring over 192 h. This suggests that DC22 is stable in ruminal fluid at a pH of 6-6.5 for extended periods of time. The dilution rate of the Rusitec (0.32 mL·min^{-1}), which represents an approximate 50–55% volume replacement in the fermentation vessels in 24 h, likely resulted in the steady decline in numbers of *E. coli* O157:H7 (Fig. 2A) and DC22 (Fig. 2B) over time.

Escherichia coli O157:H7 was detected in the feed residues and feed bags in the control fermenters 24 h post-inoculation at levels of 1.83 and 1.73 $\log_{10}$ CFU·g^{-1}, respectively. This suggests that *E. coli* O157:H7 is capable of associating for a limited time with both the fluid- and feed particle-associated microbial populations within the rumen. Numbers of *E. coli* O157:H7 recovered from the feed and feed bags subsequently declined after 24 h, with similar numbers being recovered from both feed and feed bags. Enrichment and IMS was required to recover *E. coli* O157:H7 from the feed and feed bags 72 h post-inoculation and thereafter. The organism was not recovered from the feed or feed bags

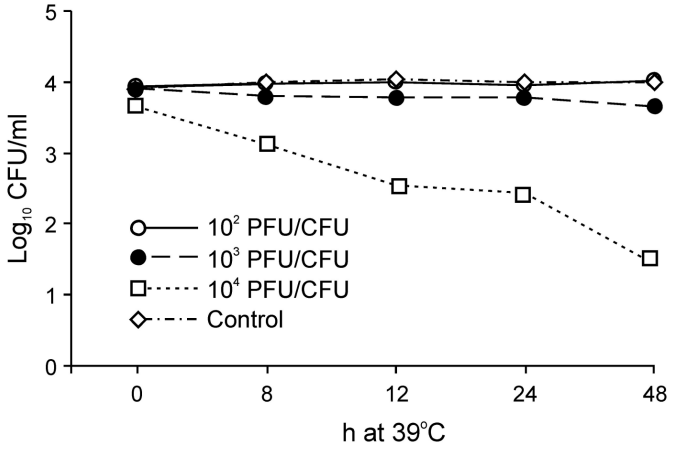

Figure 1. Recovery of *E. coli* O157:H7 strain 3081 during a 48-h incubation at 39 ºC in clarified ruminal fluid containing 10^4 CFU·mL^{-1} of *E. coli* O157:H7 strain 3081 and DC22 at 0, 10^2, 10^3 and 10^4 PFU/CFU.

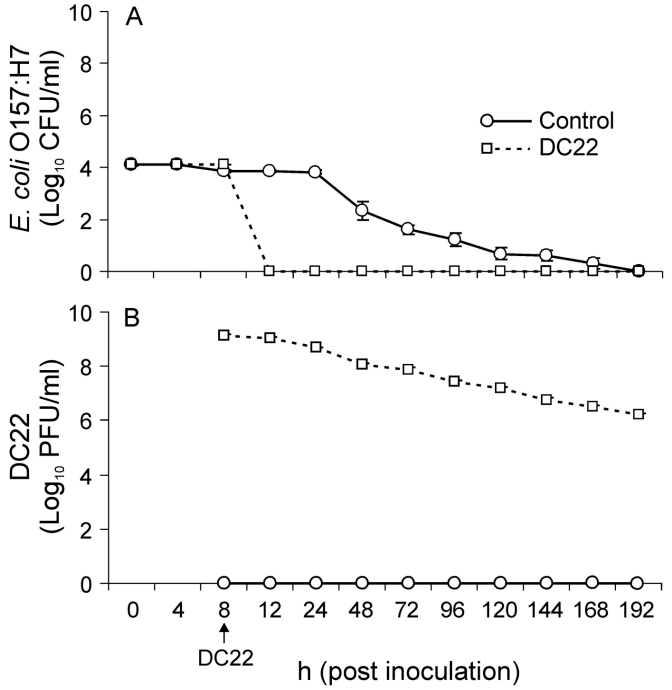

Figure 2. Recovery of (A) *E. coli* O157:H7 strain 3081 and (B) bacteriophage DC22, in ruminal fluid from Rusitec fermenters following inoculation with 10^4 CFU·mL^{-1} of *E. coli* O157: H7 strain 3081 at time 0 (control) or inoculation with 10^4 CFU·mL^{-1} of *E. coli* O157: H7 strain 3081 at time 0 and bacteriophage DC22 after 8 h at a multiplicity of infection of 10^5 PFU/CFU (DC22). Bars represent the standard error of the mean.

after 144 h post-inoculation. *Escherichia coli* O157:H7 was not detected in the feed or feed bags of the DC22-treated fermenters during the entire experimental period (data not shown). Consequently, it may require a considerable period of time after introduction for *E. coli* O157:H7 to join feed particle-associated microbial populations.

3.3. Effect of DC22 on the fecal shedding of *E. coli* O157:H7 in lambs

Preinoculation fecal samples taken from both groups of lambs were negative for *E. coli* O157:H7 using enrichment followed by IMS using Dynabeads anti-*E. coli* O157. The numbers of *E. coli* O157:H7 strain E318N shed in the feces of the lambs in both the control and DC22-treated group decreased during the first 13 d after inoculation (Fig. 3). In the control group, *E. coli* O157:H7 was not detected in the feces of five of the six lambs after 13 d. An increase in the numbers of *E. coli* O157:H7 was observed in one of the lambs 13 and 16 d post-inoculation. Numbers of *E. coli* O157:H7 then decreased in the feces of this lamb and the organism was undetectable 30 d post-inoculation (Fig. 3A). In the DC22-treated group, numbers of *E. coli* O157:H7 decreased over the first 13 d post-inoculation and then increased in three of the six lambs over the next 14 days. The organism was undetectable in the feces of all six lambs 27 d post-inoculation (Fig. 3B). Lambs in the control and the DC22-treated group were all culture negative for *E. coli* O157:H7 strain E318N 30 d post-inoculation. There was no difference ($P > 0.05$) in the numbers of *E. coli* O157:H7 shed by the lambs in the control or DC22-treated group during the 30 d experiment. Feed withdrawal from 3 lambs within treatment and control groups on days 20 and 21 had no effect ($P > 0.05$) on the subsequent fecal shedding of *E. coli* O157:H7 strain E318N on days 23, 27 or 30 (Fig. 3).

A rapid decrease in the levels of DC22 recovered from the feces of lambs was observed following administration of DC22 two days post-inoculation (data not shown). Bacteriophage DC22 was not detected in the fecal samples of any of the lambs 8 d after inoculation. Bacteriophages were not detected in the feces of the control group over the 30 d experimental period. Feed withdrawal on d 20 and 21 had no effect on the shedding of DC22 in the feces of treated lambs (data not shown).

An MOI of 10^5 PFU/CFU exerted an inhibitory effect on *E. coli* O157:H7 in the Rusitec. This high MOI indicates a low bactericidal activity of the phage or ineffective attachment of DC22 to receptors on the host cells. The bacteriophage DC22 had no effect on the fecal shedding of *E. coli* O157:H7 by lambs. In the Rusitec, an enclosed but continuous fermentation system, DC22 was likely at a sufficient MOI to result in complete lysis of *E. coli* O157:H7 within 4 h (Fig. 2A), prior to any significant reduction in numbers of DC22 or *E. coli* O157:H7 as a result of wash-out. The administration of DC22 to lambs two days post-inoculation with *E. coli* O157:H7 likely resulted in a concentration of DC22 that was insufficient to sustain lysis of *E. coli* O157:H7 within the more complex environment of the gastrointestinal tract. This may have been due to a reduction in the effectiveness of DC22 due to non-specific binding in the gastrointestinal tract along with its low bactericidal activity.

Some phages do not always lyse bacteria, but can establish a non-lytic presence in the host cell without producing any progeny virions [1]. The host cell, which is said to be lysogenic, continues to grow and divide, with replication of the phage genome or prophage being coordinated with that of the host chromosome. The prophage is maintained in the host cell by integration into the host chromosome or as an extrachromosomal plasmid [1, 3]. In a lysogenic state, the host cell resists infection by a

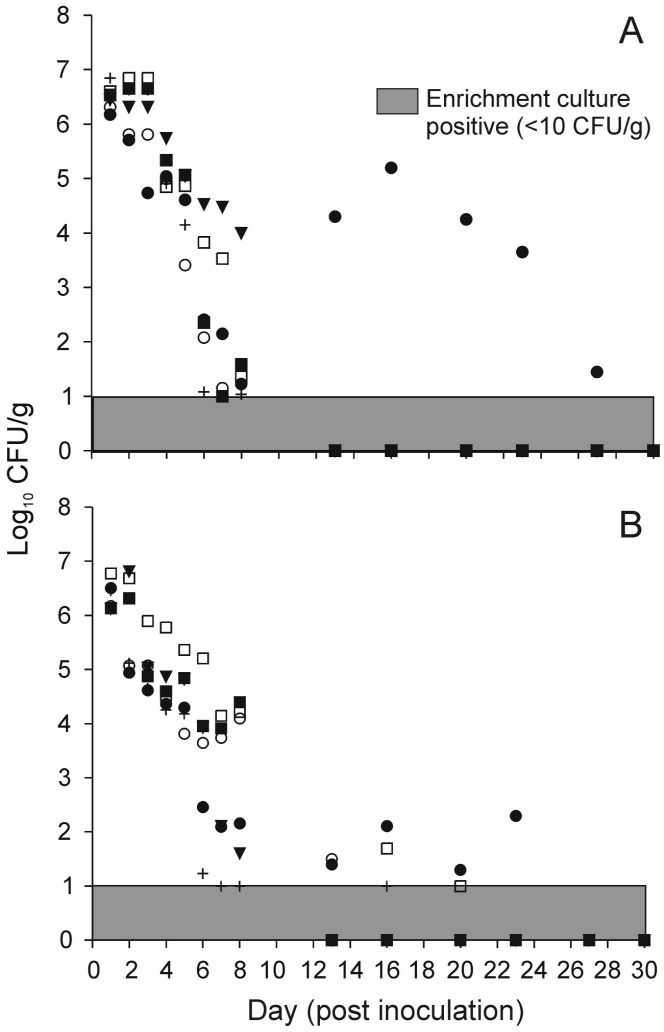

Figure 3. Levels of *E. coli* O157:H7 strain E318N shed in the feces of lambs in (A) the control group and (B) group treated with bacteriophage DC22 (N = 6 for each group). Lambs were inoculated with 10^8 CFU of *E. coli* O157:H7 strain E318N on day 0. Bacteriophage DC22 (10^5 PFU/CFU) was administered to the lambs in the treatment group on day 2. Each symbol represents an individual animal.

second phage of the same or similar type, in a process known as phage immunity. Although the capacity of a phage to lysogenize is dependent on environmental factors, it is genetically controlled. The frequency of the lysogenic response increases as the MOI increases [1]. It is possible that DC22 established a non-lytic presence in a proportion of *E. coli* O157:H7 present in the gastrointestinal tract of the lambs which resulted in a decrease in the numbers of phage particles available for infection.

Waddell et al. [34] were successful in reducing the period of *E. coli* O157:H7 fecal shedding in calves using a mixture of six *E. coli* O157-specific bacteriophages administered –7, –6, –1, 0 and 1 day following inoculation with 10^9 CFU of *E. coli* O157:H7. Smith and Huggins [29] used a mixture of two phages to protect calves, piglets and lambs from experimental *E. coli* diarrhea and reported that phage therapy was more effective when applied before or together with the target bacteria. In vitro experiments using a mixture of three O157-specific phages were successful in eliminating *E. coli* O157:H7 from cultures whereas no single phage could completely kill an *E. coli* O157:H7 culture [20]. While DC22 used alone was unsuccessful in reducing the fecal shedding of *E. coli* O157:H7 in lambs, the phage used in combination with other phages may prove effective. Optimization of the administration of bacteriophages, in terms of time of application and the use of a mixture of bacteriophages, may result in an effective and natural means to control *E. coli* O157:H7 in ruminants and their environment.

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