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In vitro studies on the colonization of bovine colonic mucosa by Shiga-toxigenic *Escherichia coli* (STEC)

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SUMMARY

This study investigated host-related factors that influence intestinal colonization by Shiga-toxigenic *E. coli* (STEC). A quantitative colonization assay was developed to comparatively measure attachment of STEC to bovine colonic tissues maintained *in vitro*. No differences were determined in colonization susceptibility between tissues derived from weaning calves and adult cattle, or for tissues from cattle fed grain and forage-based rations. Substrate conditions designed to represent various intra-enteric environments were tested for their effect on STEC/mucosal interaction. Under conditions corresponding to a well-fed ruminant (high volatile fatty acid and lactate concentrations, low pH), significantly less STEC colonized the mucosal surface of colonic biopsies. These results may help explain why fasted, poorly or intermittently fed cattle and pre-ruminant calves excrete STEC to a greater degree. Studies on the ecology of STEC within the ruminant gut help identify mechanisms to reduce their threat to public health.

INTRODUCTION

Shiga toxin-producing *E. coli* (STEC) are well-recognized human pathogens, being responsible for a range of diseases including haemolytic uraemic syndrome and haemorrhagic colitis [1]. Human clinical isolates of STEC are generally referred to as enterohaemorrhagic *E. coli* (EHEC), and *E. coli* O157:H7 is considered the classical EHEC serotype due to its significance in North America and the United Kingdom [1, 2]. Most human infections involving STEC occur via foodborne transmission, and cattle and bovine food products have been recognized as the primary reservoir for STEC [1, 2]. Some cases of EHEC disease have been linked to direct contact with cattle or their immediate environment [2].

Numerous surveys and experimental studies have investigated faecal excretion of STEC by cattle and other ruminant species [3–5]. Livestock vary in their degree of STEC excretion, whether it be the prevalence of animals shedding STEC, the faecal concentration of STEC, the duration of excretion, or the virulence of faecal STEC strains. A range of factors have been suggested to influence STEC excretion by livestock, and therefore the potential human health risk. Younger animals, particularly pre-ruminant calves, have been shown to shed STEC with a greater prevalence or in higher numbers than adult cattle [3, 5, 6]. Diet has also been linked to STEC excretion, with diets high in fibre and low in readily digestible carbohydrates associated with greater *E. coli* O157:H7 concentration and excretion duration [4, 7]. Fasting or intermittent feeding can also increase the faecal load of STEC [8–10]. Many animal management factors, such as stocking density, feeding and housing practices, and waste handling have similarly

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been proposed to influence STEC excretion [3, 5, 6]. From these studies, two main factors influencing ruminant excretion of STEC can be hypothesized: the likelihood and degree of exposure of animals to STEC, and the degree of intra-enteric carriage of STEC by individual ruminants, once inoculated. Successful colonization of the bovine gastrointestinal tract (GIT) is likely to provide a niche allowing longer term survival and reproduction, and therefore longer term shedding of higher numbers of STEC.

This study aimed to examine bovine host-related factors which may influence the colonization of the gut mucosa by STEC. An *in vitro* model was developed to comparatively measure the degree of adherence of STEC test strains to the mucosal surface of the bovine colon. Initial experiments examined the role that enteric tissue differences play in mediating STEC adherence. Because the colon represents a site of significant feed assimilation and fermentation and the physiological nature of the gut contents can vary significantly between animals depending on factors such as age and diet [11, 12], the effects of various intra-colonic environments on STEC colonization were also examined. Ionophore antibiotics are commonly added to livestock rations to improve feed conversion and control problems such as ruminal acidosis [13, 14]. As these have been suggested to influence the ecology of enteric bacteria, the effect of a common ionophore on STEC colonization was tested.

MATERIALS AND METHODS

Experimental design

Three individual experiments were performed. Experiments A and B compared STEC colonization of colonic mucosal explants that varied in terms of donor animal age and diet respectively. For Expt A, colonic biopsies were harvested from each of four adult cattle and four weaning calves. Six cattle were used for Expt B; three from a high grain ration group (grain) and three from a roughage diet group (roughage). Four explants were cultured for each animal sampled; one inoculated with each of the three STEC test strains and one uninoculated control. For Expt C, ten explants were harvested from each of three adult steers. One explant was used for each of eight substrates, a negative control explant (no STEC inoculation) and a positive control explant (maintained in standardized medium) were replicated across the three steers.

Bacterial inocula

Test strains of STEC were bovine isolates derived from the culture collection of the Food Science Australia, Brisbane Laboratory. They were EC601 (O157:H7 isolate possessing *stx₁&₂*, *eae*, *ehx*), EC743a (O26:H11; *stx₁*, *eae*, *ehx*) and EC132a (O111:H⁻; *stx₁&₂*, *eae* and *ehx*). Strains were stored at −80 °C using Protect (Technical Service Consultants Ltd, Heywood, UK) prior to subculture for 18 h at 37 °C on Luria–Bertani agar (Oxoid, Basingstoke, UK) and inoculation into the organ culture system. Bacterial cells were suspended in phosphate-buffered saline (PBS) to a standardized cell density, corresponding to a 3.0 McFarland Standard at 450 nm wavelength. A 100 μl aliquot of each bacterial cell suspension (mean standard inoculum of $6{\cdot}7\times10^{7}$ c.f.u.) was added to respective organ cultures. Inocula strains were plated onto nutrient agar (Oxoid) to confirm counts and expression of somatic antigen. EC601 was the sole inoculating strain for Expt C.

Experimental animals

Cattle in Expt A were Friesian heifers or steers. Adult cattle were 10–20 months old and weaning calves were approximately 70 days old and were fed milk and a solid starter ration at the time of slaughter. Cattle used in Expts B and C were Hereford/Shorthorn yearling steers. The high grain ration initially comprised 50% rolled sorghum and 50% feedlot meal mix (lucerne, sorghum, barley, molasses, macronutrients, no urea or antibiotics). The grain component of this ration was increased daily until it comprised 70% of feed, and was fed at this level for approximately 7 days prior to explant collection. The roughage diet was designed to represent a dry season, northern Australian pasture diet, and comprised *ad libitum* chopped spear grass (*Heteropogon contortus*) supplemented with 500 g cottonseed meal per steer per day. All animal experiments were performed following approval by the University of Queensland Animal Ethics Committee (AEC no. MICRO/PARA/138/99/UQPRS/PHD).

Quantitative colonization assay for STEC to bovine colonic mucosal explants

In vitro *organ culture* (*IVOC*)

Tissues for organ culture were harvested from experimental cattle immediately following euthanasia with

pentobarbitone sodium (Euthanasia Forte Solution; Apex Laboratories, St Mary's, Australia) or captive bolt stunning followed by exsanguination. Approximately 7 mm^2 full thickness biopsies were excised from the spiral colon via laparotomy, washed in pre-warmed PBS and transported to the laboratory in ice-cold organ culture medium (OCM). OCM was a bicarbonate-buffered 1:1 mixture of Dulbecco's modified Eagle's medium and NCTC-135 with 10% foetal calf serum and 0·5% (w/v) D-mannose (all chemicals supplied by Sigma, Castle Hill, Australia). Standardized 3 mm^2 explants were removed from biopsies using a sterile, disposable biopsy punch (Stiefel Laboratories, Castle Hill, Australia). Explants were trimmed of excessive connective tissue and placed mucosal surface uppermost on a Gelfoam sponge (Upjohn, Rydalmere, Australia). Each sponge sat within the central well of an organ culture dish (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) containing approximately 2·5 ml of pre-warmed OCM. Organ culture dishes were stacked within an airtight container and gassed with 5% CO_2/95% O_2. Organ cultures (±bacterial inoculum) were incubated at 37 °C for 8 h on a rocking platform set to 5 cycles/min. OCM was changed and re-gassed to maintain pH, nutrient and O_2 levels, and to minimize bacterial overgrowth. Residual STEC contamination of the Gelfoam allowed re-inoculation of fresh OCM with the respective test strain at each OCM change. Following incubation, explants were carefully trimmed of remaining non-mucosal tissue from the serosal surface with the aid of a dissecting microscope, rinsed gently with PBS and stored at 4 °C in a humidified microfuge tube.

Enumeration of colonizing STEC

STEC colonizing the mucosa of cultured colonic biopsies were enumerated using an indirect immunofluorescent filtration technique (IIFT). Cultured explants were placed in a phosphate-buffered digestion mixture comprising 75 U/ml Type VII high-purity collagenase (Sigma) and 5 mmol/l EDTA (Progen Industries, Brisbane, Australia) at 37 °C for 60 min. Physical homogenization was performed throughout digestion to help dislodge the intestinal epithelial lining and adherent STEC (if present) from the basement membrane. Resultant cell suspensions were centrifuged at 2300 ***g*** for 5 min, the supernatant drained, and the plug resuspended in 70% ethanol for 5 min to inactivate bacteria. The suspension was washed a further three times in PBS, with final suspension in phosphate-buffered 0·5% Tween 80 (PBT). Each cell suspension was pre-filtered through sterile glass wool to remove gross debris and made up to a final volume of 10 ml in PBT. Then 10 ml aliquots of an appropriate dilution of STEC/enterocyte cell suspension were filtered onto 25 mm diameter, 0·22 μm pore size black polycarbonate membranes (Millipore, Bedford, MA, USA) using a Bio-Foss Filtration Manifold (Foss Electric, Bishopthorpe, UK). Vacuum pressure was maintained at 0·7 bar during filtering and rinsing.

Inoculated STEC strains were identified by indirect immunofluorescent staining. Primary antibodies were supplied by Dr Roger Johnson (*E. coli* Reference Laboratory, Laboratory for Foodborne Zoonoses, Health Canada, Guelph, Ontario). Sera raised in rabbits against heat-killed *E. coli* O157:H7, *E. coli* O26:H11, and *E. coli* O111:NM were filter sterilized and adsorbed with serogroups with which they were known to cross react. Membranes were incubated with 1:1000 dilutions of the respective primary antisera using standard methods and rinsed three times with 5 ml PBT. FITC-conjugated anti-rabbit secondary antibodies (Dako Corporation, Carpinteria, CA, USA) were then applied, and membranes incubated and rinsed as for the primary antisera. Negative control explants were incubated with a mixture of O157, O26 and O111 primary antibodies, each at 1:1000 dilution. Membranes were mounted on glass slides prior to counting bacterial cells. Cell counting was performed with an epifluorescent microscope (BH-RFL; Olympus, Tokyo, Japan) incorporating a short-wave pass band excitation filter (455 nm) and a dichroic mirror. Only objects that fluoresced green and were unmistakably bacillus-shaped were enumerated. Between 200 and 250 cells were counted, the average number of cells/field derived, and a colonizing STEC count per explant calculated using dilution and magnification factors.

Simulation of enteric substrate conditions

Substrates designed to represent different intra-colonic physiological environments were added to OCM in the following manner:

(1) Low-concentration volatile fatty acids (50 mM VFA): acetic acid, propionic acid and butyric acid (A:P:B, British Drug Houses, Poole, UK) were added in molar proportions of 65:25:10 respectively to provide a combined concentration

of 50 mM. High concentration VFA (120 mM VFA): as per low VFA, although combined to provide a final concentration of 120 mM.

(2) VFA composition representing a forage-based diet (Forage VFA): A:P:B added in molar proportions of 80:15:5 to provide a final concentration of 120 mM.

(3) VFA composition representing a grain-based diet (Grain VFA): A:P:B added in molar proportions of 55:30:15 to provide a final concentration of 120 mM.

(4) Lactic acid (Lactate): an equilibrated mixture of D-lactic and L-lactic acids (British Drug Houses) to provide a combined concentration of 15 mM.

(5) Ammonia (Ammonia): 28% w/v ammonia solution (Sigma) at 15 mg/dl.

(6) Ionophore (Monensin): monensin sodium (Sigma) at 4 mg/l.

(7) Low pH (pH 6): OCM pH was reduced from 7·4 to 6·4 by addition of HCl. All preceding media were pH neutralized (pH 7·4) using NaOH or HCl following the addition of respective substrates.

Statistical analysis

Statistical comparisons of STEC colonizing counts between animals and between serotypes were performed using ANOVA incorporating a balanced design for Expt A, and a general linear model for Expts B and C (Minitab Inc., State College, PA, USA).

RESULTS

Expt A

Colonizing STEC counts for each serotype and each explant inoculated are presented in the Table. Mean STEC colonizing counts for adult cattle and weaning calf explants were calculated for each serotype and as means for the whole experiment (Fig. 1). Calf explants were not colonized by STEC in significantly greater numbers than the adult cattle explants ($P=0{\cdot}365$).

Expt B

The STEC colonizing counts for each explant are incorporated within the Table. Mean counts for each serotype of STEC and the mean STEC colonization count are presented in Figure 2. There was no significant difference in STEC colonization between tissues derived from cattle on high grain or high roughage rations ($P=0{\cdot}962$).

Table. *Colonizing counts (log_{10}) for STEC of serotype O157, O26 or O111 to bovine colonic explants cultured* in vitro

Explant	O157	O26	O111	NC*
Adult 1	6·590	6·454	6·237	0
Adult 2	6·871	4·986	6·298	4·322
Adult 3	6·166	6·234	6·460	0
Adult 4	6·175	4·694	6·295	0
Weaner 1	6·451	5·230	6·558	0
Weaner 2	6·937	6·600	6·084	0
Weaner 3	6·225	6·189	6·346	0
Weaner 4	6·540	6·447	6·064	0
Roughage 1	6·945	6·303	5·901	0
Roughage 2	6·292	6·113	5·084	0
Roughage 3	6·572	6·097	5·825	0
Grain 1	6·879	6·291	5·869	0
Grain 2	5·918	6·121	5·549	0
Grain 3	6·505	5·505	6·327	0
Mean	6·497	5·916	6·051	

* Negative control, the combined count for serotypes O157, O26 and O111 on control explants.

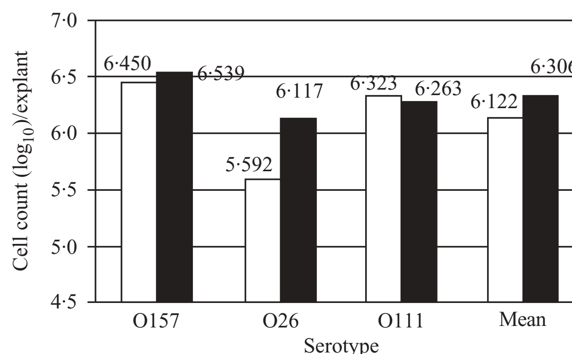

Fig. 1. Comparison of mean STEC (serotypes O157, O26, O111) colonizing counts between explants from adult cattle (□) and weaning calves (■). Counts above bars are the means for the three serotypes.

Using the combined data from the above experiments, the mean colonizing log counts for the O157, O26 and O111 inoculating strains were 6·497, 5·916 and 6·051 respectively. The colonizing count for strain EC601 (O157) was significantly greater than the counts for EC743a (O26) and EC132a (O111) using Tukey's pairwise comparisons in a one-way ANOVA ($P=0{\cdot}002$).

Expt C

Colonizing counts for EC601 to bovine colonic explants under the different substrate conditions for each of the three experimental cattle and mean counts

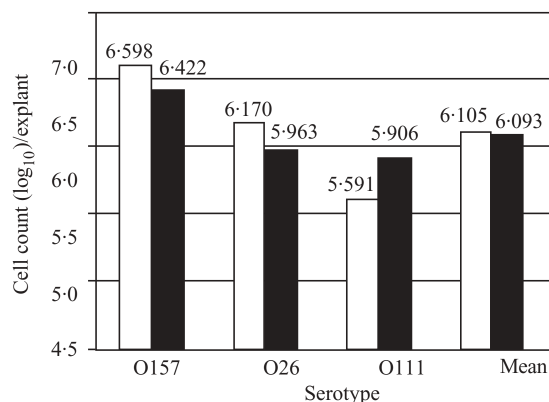

Fig. 2. Comparison of mean STEC colonizing counts between explants from cattle on high grain (■) or high roughage (□) diets for serotypes O157, O26, O111 and the combined serotype mean.

are presented in Figure 3. Generally, the presence of the various substrates reduced the colonization of the colonic mucosa by EC601 compared to the positive control, though significant decreases were only noted with the presence in OCM of 120 mM VFA, Forage VFA, Grain VFA (95% confidence level) and the presence of lactate (90% confidence level). The only substrate which seemed to have no appreciable effect on colonization was monensin. Only one negative control explant was found to have *E. coli* O157:H7 present, the count being $1{\cdot}6 \times 10^2$.

DISCUSSION

In this study, a quantitative colonization assay was used to compare the association of STEC with bovine colonic mucosal tissues under a range of host-related factors. Differences in the ability of STEC to colonize the GIT of cattle is likely to contribute to the patterns of STEC excretion seen in ruminants experimentally and in the field. These patterns particularly relate to the age and diet of ruminants. Successful colonization of the GIT of an animal by a particular organism generally relies on some degree of mucosal association in order to avoid rapid excretion of that organism with the gut contents [15, 16]. Colonic tissues were chosen for colonization testing, as the large bowel is generally considered the primary habitat for *E. coli*, whether they reside within the luminal contents or are associated with the mucosal surface [17].

The role of host tissue susceptibility to colonization was examined initially using organ-cultured tissues from animals of different age and dietary backgrounds, while the bacterial strains and conditions in which the bacteria and mucosa interacted were kept constant. Several mucosally associated mechanisms are suspected to mediate adherence of STEC to the enteric surface. A range of receptors exist for bacterial adherence factors such as fimbriae [17], intimin [18, 19], and other adhesins [20]. These receptors vary in their number or affinity between neonatal and adult animals, resulting in age-associated susceptibilities to enteric colonization and excretion [21]. Differences in the presence and action of mucosal immunological features in pre-ruminant and adult ruminant animals might also explain an increased susceptibility to STEC carriage by calves. This may relate to secretory immunoglobulins (sIgA) acquired by colostrum intake [19], the development of specific antibodies against adherence factors [22], or cytokine responses to mucosal STEC exposure [23].

However, no significant differences were noted in the numbers of STEC colonizing mucosal tissues from weaning and adult cattle, or from cattle fed either forage or grain-based diets. It is possible that mucosal tissues from weaning calves are not significantly different structurally or functionally to those of adult cattle. Further comparisons between pre-weaned calves, weaning calves and adult cattle and susceptibility to STEC colonization are required to investigate this possibility. Limitations in the IVOC technique may be responsible for the lack of colonization variation between tissues. For instance, IVOC cannot model *in vivo* GIT effects such as gut passage rates or digesta consistency. Another possibility is that the interaction of STEC and the intestinal mucosa is influenced by features other than those relating specifically to the bacterium or the tissues themselves. The intra-enteric environment was proposed to play a critical role in determining an animal's susceptibility to colonization by STEC. In order to examine this possibility, the effects of different physiological conditions that represent age or diet effects on the contents of the gut were examined. In Expt C, STEC strain and mucosal tissues were kept constant, while a variety of substrates (VFA, lactate, etc.) were used to represent different intra-intestinal environments.

A substrate of key interest within this study was VFA. The production of VFA in the rumen increases relative to the amount of readily digestible feed available and the same dietary effects have been noted for VFA concentrations within the distal gut [11, 24]. Steers fed large amounts of grain demonstrated caecal VFA levels 80% higher than those fed forage [12]. Diet also affects the composition of VFA in the rumen and distal gut, mainly through variations in the molar

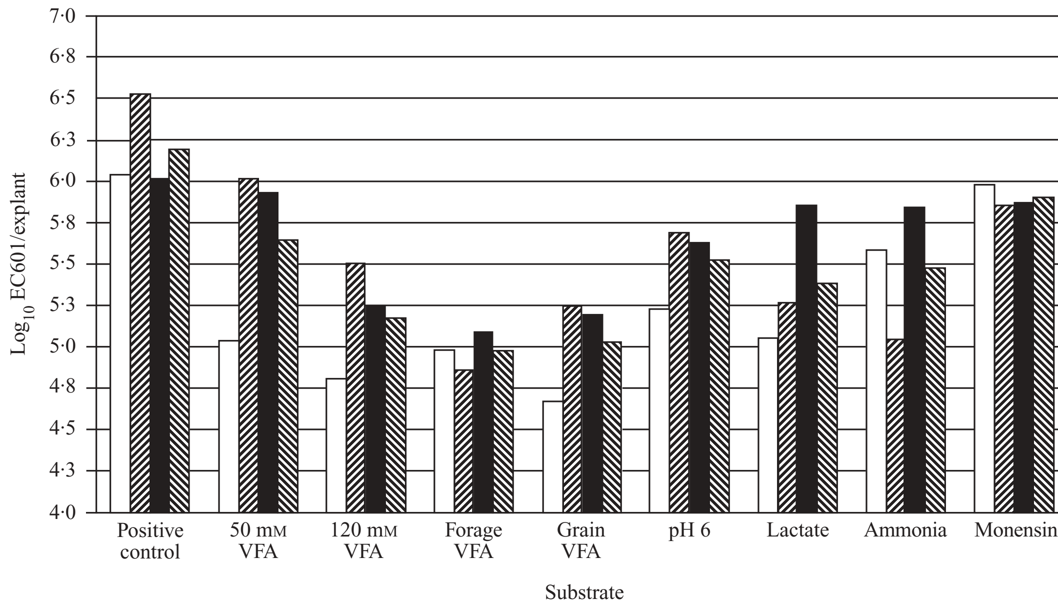

Fig. 3. Colonizing counts for *E. coli* O157:H7 (EC601) to bovine colonic explants maintained *in vitro* under a variety of substrate conditions. Results are shown for steer 1 (□), steer 2 (▨), steer 3 (■) and the mean of the three steers (▧).

proportions of acetate, propionate and butyrate (A:P:B). Where cattle or sheep are fed readily digestible feeds such as grains or chopped forage compared to high fibre rations, ruminal acetate levels decrease, and propionate and butyrate ratios increase [25]. The same physiological changes in the intra-enteric environment occur within the caecum and large intestine with grain *vs.* forage feeding [11, 12, 24]. Similarly, concentrations of lactate are higher within the caeca and colons of ruminants given feeds high in readily fermentable substrates, particularly grains [11, 14]. Such dietary differences also influence the pH of gut contents in ruminants. Cattle and sheep fed high grain rations have ruminal and distal gut pH levels 1–2 points lower than those of roughage-fed animals [11, 24, 25]. Much of the pH effect is due to changes in intestinal VFA and lactate concentrations [24]. Faecal pH and VFA and lactate concentrations can vary also with the type of grain fed, due to relative levels of fermentable carbohydrate in the hindgut [12, 14].

The gastroenteric levels of these key substrates (pH, VFA, lactate) have also been shown to vary in ruminants depending on age. Pre-weaned ruminants do not ferment large quantities of plant material within their gut compared to adult or weaning ruminants, resulting in reduced VFA concentrations within the rumen and hindgut [26]. VFA composition also changes with age, with a decrease in the molar proportion of acetate being the most evident feature as calves and sheep commence functional rumination [26, 27]. Although lactate concentrations within the GIT of calves prior to the introduction of solid feed are much higher than weaned calves [14, 26], the decreased VFA concentration results in generally lower intra-enteric pH conditions in ruminating cattle than calves [27].

The presence of VFA had a significant effect on STEC/mucosal interaction *in vitro*. A reduction in colonization was evident with high concentrations of VFA (120 mM), irrespective of VFA composition. This suggests that under conditions where large amounts of VFA are being produced in the hindgut, there may be a reduction in STEC adherence to the gut wall, and therefore a potential reduction in STEC carriage. Conditions favouring increased production of VFA include the feeding of high-quality diets, particularly grains, and may help to explain why STEC shedding in these animals is often found to be reduced compared to forage-fed ruminants [4, 7]. Similarly, it may be the reason that animals on a low plane of nutrition or fasted tend to have concurrent increases in STEC shedding [9]. Rasmussen et al. [8] and Harmon et al. [10] proposed that under conditions of dietary stress, *E. coli* O157:H7 may be able to successfully occupy the enteric niche and therefore be shed in greater numbers. Similarly, Kudva and colleagues [4] suggested that a drop in VFA concentration was likely to be the mechanism by which diets high in fibre and low in nutrients allowed a greater and more prolonged shedding of *E. coli* O157:H7 by sheep. The current study suggests that

a possible reason STEC are shed in greater numbers or for longer durations by animals poorly or intermittently fed is because they are better able to colonize the gut mucosa, thereby allowing continued habituation and reproduction in this niche. This phenomenon may also explain why pre-ruminant calves tend to excrete higher levels of STEC or *E. coli* O157:H7 in their faeces [5, 8].

Through physiological modifications of the bacterial/mucosal interaction mechanisms mentioned above, age and dietary differences between ruminants may affect the potential for their intestinal tract to be colonized by STEC. *E. coli* expression of fimbriae, particularly of bovine F5 (K99) pilus, is affected by a many factors, including pH, glucose concentration or the presence of amino acids [28, 29]. Expression of intimin by enteropathogenic *E. coli* has been similarly demonstrated to be influenced by environmental factors during bacterial growth and following attachment and effacement lesion formation [30]. While less is known about the regulation of intimin expression in STEC [19], it has been suggested that mucosal adherence may be modulated by the effects of various intra-luminal substrates on the action of intimin or other outer membrane adherence mechanisms [31]. Such effects may also be attributed to differences in expression or activation of fimbrial or intimin receptors. Less specific mechanisms of bacterial attachment to intestinal mucosa may also be affected by enteric environmental conditions. The mucous layer represents an important colonization location for *E. coli*, and can be altered by differences between individuals in terms of age, diet or the presence of various substrates [15, 32]. Changes in parameters such as pH and VFA concentration also affect the rate of enterocyte turnover within colonic crypts of cattle and humans [33, 34]. By changing the rate of shedding of epithelial cells (and associated STEC-binding sites) into the lumen of the gut, diet and age may modify the likelihood, degree or duration of STEC excretion in the faeces.

While high intra-enteric concentrations of ammonia have been demonstrated to modify the nature and function of the mucosal surface [33] or the surface expression of intimin [30], the presence of ammonia in OCM had no significant effect on STEC colonization of colonic mucosa *in vitro*. Similarly, the presence of an ionophore antibiotic did not seem to affect STEC/mucosa interaction, despite the demonstrated effects on VFA concentration and composition [13, 14]. It is possible that these substrates rely on the concurrent presence of other physiological changes to act on the mechanisms of bacterial colonization. This phenomenon was not accounted for in the current study, as each substrate was assessed individually. Further investigations using the currently described technique should aim to combine various substrate conditions in order to more accurately represent specific intra-enteric environments. For instance, the combined effects of reduced pH, elevated VFA concentration and elevated lactate levels may act synergistically in reducing the degree of STEC colonization of the ruminant intestine. Further studies using this *in vitro* model may also be useful in examining the colonizing abilities of different STEC strains. In this study, the O157:H7 strain colonized bovine colonic mucosal tissues in significantly greater numbers than the O26 or O111 strains.

Once determined, the mechanisms that govern the interaction of STEC and the enteric surface may be exploited to reduce the carriage of STEC within the GIT, and thereby reduce the degree or duration of faecal excretion of this potential pathogen by livestock. It is likely that these mechanisms will involve the bacterium itself, the host tissues and the environment in which the two interact. Of these, modification of intra-enteric physiological conditions represents a relatively practical means by which STEC excretion may be altered. Studies that examine the roles of diet, age, species and other factors on the excretion of foodborne pathogens by livestock are required in order to create animal management protocols that reduce zoonotic risk.

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