

## Intermittent *Escherichia coli* O157:H7 colonisation at the terminal rectum mucosa of conventionally-reared lambs<sup>§</sup>

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**Abstract** – In cattle, the lymphoid rich regions of the rectal-anal mucosa at the terminal rectum are the preferred site for *Escherichia coli* O157:H7 colonisation. All cattle infected by rectal swab administration demonstrate long-term *E. coli* O157:H7 colonisation, whereas orally challenged cattle do not demonstrate long-term *E. coli* O157:H7 colonisation in all animals. Oral, but not rectal challenge of sheep with *E. coli* O157:H7 has been reported, but an exact site for colonisation in sheep is unknown. To determine if *E. coli* O157:H7 can effectively colonise the ovine terminal rectum, in vitro organ culture (IVOC) was initiated. Albeit sparsely, large, densely packed *E. coli* O157:H7 micro-colonies were observed on the mucosa of ovine and control bovine terminal rectum explants. After necropsy of orally inoculated lambs, bacterial enumeration of the proximal and distal gastrointestinal tract did suggest a preference for *E. coli* O157:H7 colonisation at the ovine terminal rectum, albeit for both lymphoid rich and non-lymphoid sites. As reported for cattle, rectal inoculation studies were then conducted to determine if all lambs would demonstrate persistent colonisation at the terminal rectum. After necropsy of *E. coli* O157:H7 rectally inoculated lambs, most animals were not colonised at gastrointestinal sites proximal to the rectum, however, large densely packed micro-colonies of *E. coli* O157:H7 were observed on the ovine terminal rectum mucosa. Nevertheless, at the end point of the study (day 14), only one lamb had *E. coli* O157:H7 micro-colonies associated with the terminal rectum mucosa. A comparison of *E. coli* O157:H7 shedding yielded a similar pattern of persistence between rectally and orally inoculated lambs. The inability of *E. coli* O157:H7 to effectively colonise the terminal rectum mucosa of all rectally inoculated sheep in the long term, suggests that *E. coli* O157:H7 may colonise this site, but less effectively than reported previously for cattle.

***E. coli* O157:H7 / IVOC / lymphoid / terminal rectum / ovine**

### 1. INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is a major cause of human

gastrointestinal disease that can lead to life-threatening illnesses, such as haemorrhagic colitis and renal failure [13, 24]. The main transmission routes for human infection are through faecal contamination of foods and direct animal contact. Ruminants, especially

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cattle, are cited as the main reservoir, although small ruminants, such as sheep, have also been implicated [2, 3, 9, 14, 23].

As a necessity for the development of optimal intervention strategies, various ruminant studies, mostly in cattle, have reported *E. coli* O157:H7 tissue tropisms in the ruminant gastrointestinal tract (GIT). Indeed Grauke et al. [12] first suggested that the lower GIT of orally inoculated cattle and sheep, including the colon, was one location associated with *E. coli* O157:H7 persistent colonisation. More recently, Naylor et al. [23] showed the lymphoid follicle-dense mucosa at the terminal rectum, as the primary site for *E. coli* O157:H7 localisation in young calves following oral inoculation. This hypothesis was further assessed by Sheng et al. [26] who demonstrated that rectal swab administration of *E. coli* O157:H7 resulted in persistent long-term colonisation, thus strongly supporting the terminal rectum mucosa as the preferred site for *E. coli* O157:H7 colonisation in cattle. A recent feedlot *E. coli* O157:H7 infection study generated supportive data suggesting that “super-shedder” status is linked to greater *E. coli* O157:H7 colonisation of the terminal rectum mucosa [5]. Furthermore, necropsy of cattle that were defined as long-term colonised by *E. coli* O157:H7, revealed that of all GIT sites examined, only the terminal rectum was culture positive for *E. coli* O157:H7 [17]. Collectively, these data are compelling that in cattle, *E. coli* O157:H7 colonises the lower GIT effectively, but that long term and high level shedding is dependent upon colonisation of the terminal rectum mucosa.

Significantly less is known about the primary site of colonisation for *E. coli* O157:H7 in sheep, should a single site exist. Interpretation of data from the various oral inoculation studies conducted [6, 7, 12, 15, 28] have not identified the terminal rectum as the primary site of colonisation. Indeed, small sparse attaching and effacing (A/E) lesions induced by *E. coli* O157:H7 have been identified in the ovine colon more readily than at other sites [28]. Using the same *E. coli* O157:H7 isolate, our and other laboratories have investigated and hypothesized the role

of various *E. coli* O157:H7 virulence factors in the well established conventionally weaned 6-week-old lamb model [15, 27, 29]. Indeed, the *E. coli* O157:H7 isolate (NCTC12900nal<sup>r</sup>) previously reported from orally challenged 6-week-old lambs by our laboratory has demonstrated persistent shedding in the faeces [15, 28], but detailed bacterial enumeration of the ovine gastrointestinal tract has not been undertaken to determine if the terminal rectum is preferentially colonised. For the study to be presented here, in vitro organ culture (IVOC) was initiated to determine if our *E. coli* O157:H7 isolate could colonise the ovine terminal rectum mucosa. Since *E. coli* O157:H7 rectal inoculation of cattle did demonstrate long-term colonisation in all experimentally infected animals, comparative rectal and oral inoculation studies that encompassed detailed characterisation of the ovine gastrointestinal tract were performed to determine if indeed the terminal rectum was the primary localisation site associated with *E. coli* O157:H7 persistence in the ovine host.

## 2. MATERIALS AND METHODS

### 2.1. Bacterial strain, inocula and reagents

*E. coli* O157:H7 strain NCTC12900nal<sup>r</sup> is a naturally occurring strain and was previously described and characterised using relevant in vitro and in vivo models [1, 15]. It does not possess *stx1* and *stx2*, but it does induce attaching and effacing (A/E) rearrangements on HEp-2, and porcine cell lines and in vivo ovine models. NCTC12900nal<sup>r</sup> was stored in heart infusion broth (HIB) supplemented with 30% glycerol (v/v) at -80°C and was streaked to single colonies on 5% sheep blood agar when required. For bovine and ovine IVOC assays and ovine in vivo studies, bacterial inocula was prepared by growth in 20 mL or 100 mL LB broths for 16 h, at 37°C with shaking (225 rpm). For IVOC assays, 25 µL of overnight culture, corresponding to approximately  $2 \times 10^7$  CFU, was inoculated directly onto each tissue explant. For in vivo studies, overnight LB broth cultures, corresponding to approximately  $1 \times 10^9$  CFU/mL ( $1 \times 10^{10}$  inocula) were administered as described below.

## 2.2. Experimental animals

For ovine and bovine IVOC, two healthy 6-week-old cross-bred lambs and two 12-week-old Holstein bull calves were used on two separate occasions. For ovine in vivo studies, a total of thirty, healthy 5-week-old cross bred lambs were used. Lambs were separated into groups and each group was housed in a separate room with a cement floor, maintained at negative pressure. For experiment 1, 12 5-week-old cross-bred conventionally reared lambs were randomly separated into two equal groups (A and B). For experiment 2, 18 cross-bred conventionally reared 5-week-old lambs were randomly divided into two groups, group C comprising of twelve animals and group D comprising of six animals. All groups were adapted to feed and housing conditions for at least one week prior to *E. coli* O157:H7 inoculation. Throughout these studies all lambs were fed pelleted feed twice daily and had free access to drinking water. Prior to challenge faecal samples were taken from all lambs and cattle used in all studies and confirmed negative for *E. coli* O157:H7 by immunomagnetic separation (IMS) and culture.

## 2.3. Bovine and ovine IVOC

The bovine and ovine intestinal IVOC system was adapted from the bovine and porcine intestinal IVOC systems described previously [10, 11]. Briefly, the rectal anal tissue of the terminal rectum and the last 2 cm of the rectum immediately before the terminal rectum, termed rectum-1, were excised and placed in cold complete RPMI medium without D-mannose. Approximately 12 mm<sup>2</sup> explants were biopsied and placed mucosal side up onto biopsy foam pads, in sterile 24-well Nunclon Delta surface tissue culture plates, with 4 explants on each biopsy foam pad. Complete RPMI 1640 medium (10% Fetal Bovine serum, 0.25% lactalbumin hydrolysate, 0.2 µg/mL hydrocortisone, 0.1 µg/mL insulin, 75 mM β-mercaptoethanol, 2 mM L-glutamine, 2 mM L-aspartate) was added to each well (1 mL). Explants were then placed on a see-saw rocker and maintained at 37 °C (5% CO<sub>2</sub>) for 8 h. Complete RPMI medium was replaced after the first 2 h post incubation and hourly thereafter.

## 2.4. In vivo challenge methods and collection of faeces

Two separate in vivo studies were performed with lambs challenged at 6-weeks of age. For

experiment 1, all lambs in group A were orally challenged with  $1 \times 10^{10}$  CFU of *E. coli* O157:H7 isolate NCTC12900nal<sup>r</sup>. Inocula were delivered in a 10 mL volume using a worming gun (Novartis Animal Health) ensuring that the whole inoculum was delivered directly to the pharynx. Group B lambs acted as a negative control for analysis of immune cell populations in the epithelial cells of the terminal rectum. Rectal faecal samples were taken by digital insertion from all lambs in group A on days 3, 5 and 7 after inoculation. For experiment 2, all lambs in group C were dosed per rectum following manual faecal evacuation with  $1 \times 10^{10}$  CFU of *E. coli* O157:H7. Inocula were delivered in a 10 mL volume using a syringe and flexible catheter tube (Agrihealth, lamb reviver). Lambs were restrained for 2 min to ensure the inocula remained in the rectum. All group D lambs were orally challenged with  $1 \times 10^{10}$  CFU of *E. coli* O157:H7 as described for groups A and B of experiment 1. Faecal samples were taken per rectum by digital insertion daily for fourteen days post inoculation. For both experiment 1 and 2, necropsies were performed on selected lambs (Sect. 2.5). All procedures were conducted under the jurisdiction of Home Office licence 70/6250 granted under the Animals (Scientific Procedures) Act (1986).

## 2.5. Necropsy of *E. coli* O157:H7 infected lambs

All lambs from groups A and B from experiment 1 and from group C of experiment 2 were euthanased and necropsied as described previously [6]. Briefly, for experiment 1, two lambs were examined at days 3, 5 and 7 after inoculation, respectively. Tissue samples, either for bacterial enumeration or immunohistochemistry (IHC), were collected from the duodenum, jejunum, ileum, caecum, ascending colon, spiral colon, the last 2 cm of the distal rectum and the terminal rectum. For experiment 2, three lambs from group C were examined at day 1 and day 3 after inoculation and the remaining six animals were examined on day 14 after inoculation. Tissue samples for bacterial enumeration, IHC or confocal analysis, were collected from the duodenum, jejunum, ileum, caecum, ascending colon, spiral colon, terminal rectum and from six sites excised at approximately 2 cm apart measured from the terminal rectum (rectum-1), along the rectum toward the distal colon (rectum-2 to rectum-6) [15]. Group D lambs did

not undergo necropsy since bacterial enumeration data for orally challenged animals was collated in experiment 1. However, an orally challenged group was still required for experiment 2, firstly, as a control for *E. coli* O157:H7 recovery in faecal samples and secondly, all six animals were required for effective statistical analyses.

## 2.6. Bacterial enumeration

For experiments 1 and 2, all *E. coli* O157 isolates were detected using methods described previously [15]. Briefly, lamb faeces (1 g) and necropsy tissue samples (1 g) were separately re-suspended in 9 mL of BPW, mashed with sterile forceps and vortexed. Ten-fold-serial dilutions were plated directly onto sorbitol MacConkey agar (SMAC) plates supplemented with nalidixic acid (15 µg/mL). If no direct counts were observed after overnight incubation of the plates at 37 °C, the BPW homogenates were enriched by incubation at 37 °C for 6 h, and then plated onto SMAC supplemented with nalidixic acid (15 µg/mL). The serogroup of bacteria recovered was verified by *E. coli* O157-specific latex agglutination (Oxoid, Basingstoke, UK).

## 2.7. Microscopy

Preparation of tissues taken at necropsy for subsequent visualisation by light and confocal microscopy was performed as described previously [15].

## 2.8. Statistical analysis

For oral and rectal shedding data from in vivo experiment 2, analysis of variance (ANOVA) was calculated on the log<sub>2</sub> (counts). The data was used to calculate the difference between the bacterial counts for the oral and rectally *E. coli* O157:H7 inoculated lambs for each day and a *p*-value and 95% confidence intervals were generated.

## 3. RESULTS

### 3.1. Interaction of *E. coli* O157:H7 NCTC12900naI<sup>r</sup> with ovine IVOC

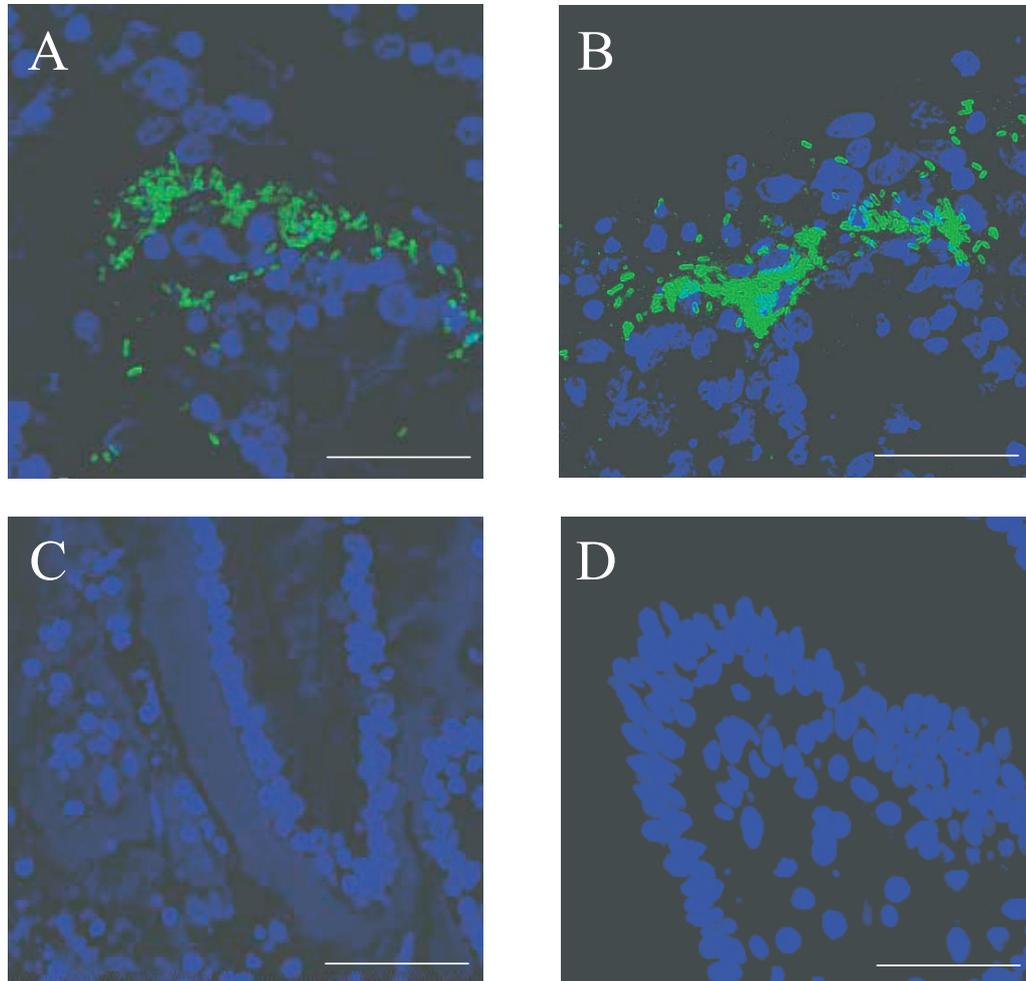
The ability of *E. coli* O157:H7 NCTC12900naI<sup>r</sup> to associate intimately with ovine and bovine rectum-1 and terminal rectum explants was assessed. After staining

with anti-O157-FITC and analysis by confocal microscopy, small clusters of *E. coli* O157 (< 10 bacteria) were observed, although diffuse adherence patterns were also noted throughout all explant types. Albeit sparsely, larger, more densely packed micro-colonies of *E. coli* O157 were associated with the mucosa of terminal rectum explants (Fig. 1), but not the mucosa of rectum-1 explants (data not shown). Similar findings were made for the bovine control tissues.

### 3.2. Localisation of *E. coli* O157:H7 in the ovine gastrointestinal tract after oral inoculation of lambs

For experiment 1, lymphoid and non-lymphoid intestinal tissues from two, randomly selected orally inoculated 6-week-old lambs were taken at necropsy on days 3, 5 and 7 after oral inoculation. Samples were analysed by bacterial enumeration and tissues were also stained for CD3 (T-cell), CD79a (B-cell) and CD68 (monocyte macrophage) cells. Bacterial enumeration of homogenised tissues removed at necropsy did reveal that although all lambs were *E. coli* O157:H7 positive, not all lambs were positive for every tissue cultured (Fig. 2). Furthermore, there was variation in the numbers of *E. coli* O157:H7 recovered from each lamb at each time point. However, on days 5 and 7 after inoculation, approximately a 100-fold more *E. coli* O157:H7 were recovered from the rectum-1 and terminal rectum sites compared with all other GIT sites cultured. Comparison of lymphoid and non-lymphoid tissue was inconclusive with higher numbers recovered from lymphoid tissues in lambs C and F and from non-lymphoid tissues in lambs D and E. The highest numbers of *E. coli* O157:H7 were recovered consistently from the faeces of lamb D.

Analysis of immune-cell infiltration in all lambs from days 5 and 7 after inoculation, revealed similar levels of T-cell, B-cell and monocyte macrophage specific staining in terminal rectum associated lymphoid tissue of lambs infected with *E. coli* O157:H7 as in unaffected controls (data not shown).

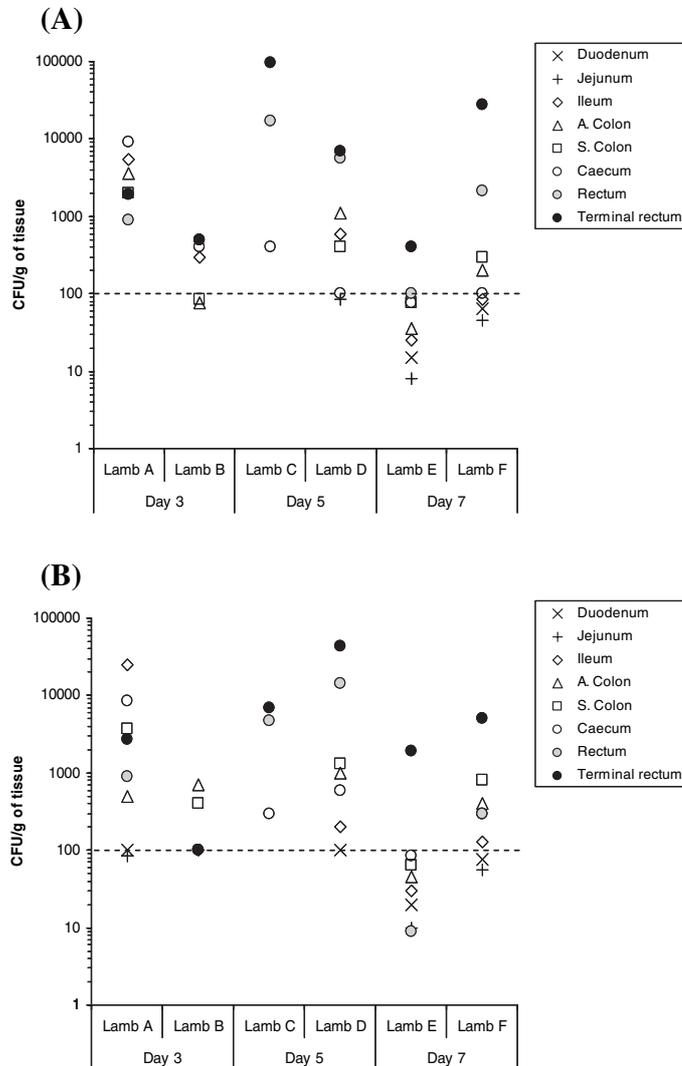


**Figure 1.** Observation of *E. coli* O157:H7 densely packed micro-colonies on ovine (A) and bovine (B) terminal rectum explants as visualised by confocal microscopy. Dapi (blue) = nuclei. FITC (green) = *E. coli* O157. (C) and (D) are uninfected ovine and bovine terminal rectum explants, respectively. *E. coli* O157:H7 was incubated with explants for 8 h with changing of the media occurring every hour after the first 2 h. Bar (A) = 32.17  $\mu\text{m}$ , (B) = 27.5  $\mu\text{m}$ , C = 49.26  $\mu\text{m}$ , D = 31.77  $\mu\text{m}$ .

### 3.3. Duration and extent of *E. coli* O157:H7 faecal shedding from lambs inoculated rectally and orally

For experiment 2, faecal samples were taken daily over a 2-week period from 6 orally and 6 rectally inoculated 6-week-old lambs, and plated onto the appropriate media. The faecal shedding data from each individual lamb are shown in Figure 3. Twenty-four

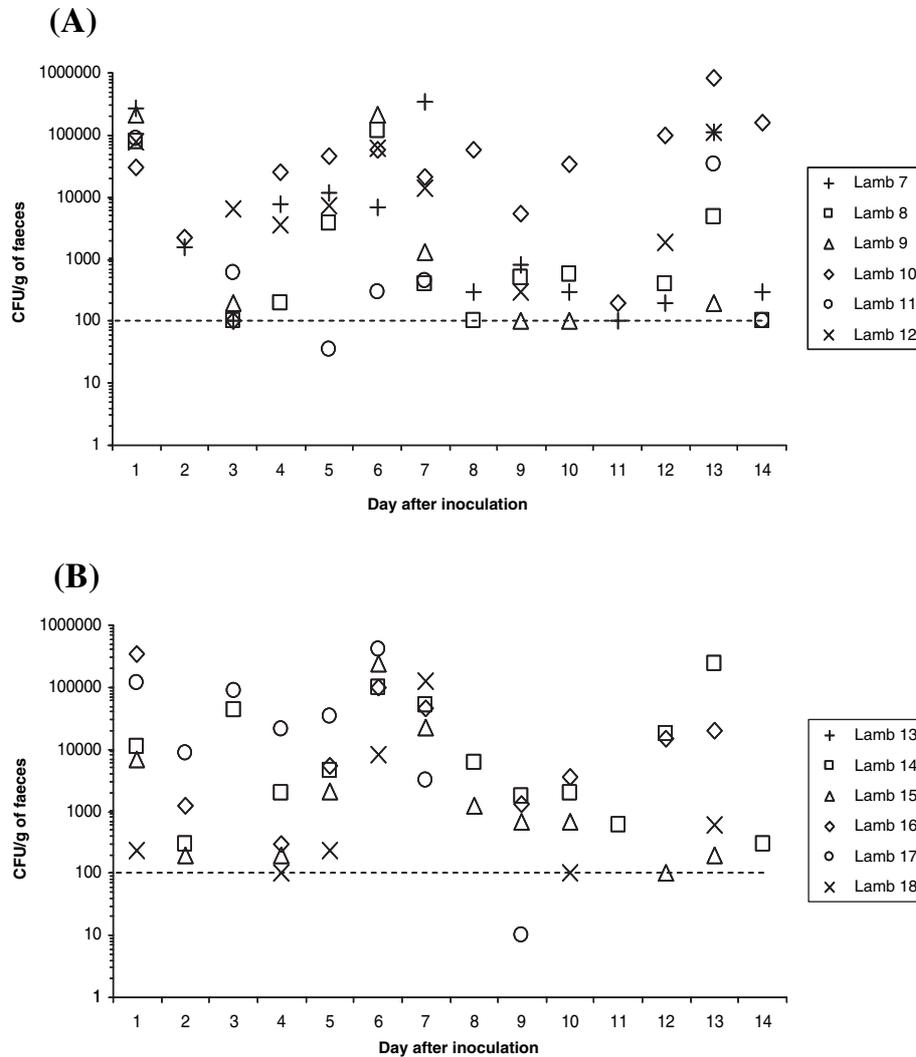
hours after rectal and oral inoculation, 6 and 5 lambs were positive, respectively, with recoveries at  $10^4$ – $10^5$  and  $10^2$ – $10^5$  CFU/g faeces<sup>-1</sup>, respectively. Throughout the 2-week period of the study, variable shedding of *E. coli* O157:H7 was noted with only one significant difference ( $p = 0.044$ ) between groups observed at day 1 after inoculation in which recovery of *E. coli* O157:H7 from animals inoculated rectally was higher. Over the



**Figure 2.** Evaluation of *E. coli* O157:H7 tropisms to lymphoid (A) and non-lymphoid (B) regions of the GIT in young lambs orally inoculated with approximately  $1 \times 10^{10}$  CFU, as determined by bacterial enumeration after necropsy. All data points on or above the dotted line were obtained after direct plating. All data points below the dotted line were obtained after enrichment of faecal samples negative for growth after direct plating. These enrichment data points are only nominal and could represent any value between 1 and 99 CFU/g faeces<sup>-1</sup>. Samples where no *E. coli* O157:H7 growth was detected after enrichment are not shown. N.B.: Non-lymphoid ileum was actually sampled from a part of the ileum where less dense lymphoid tissue reside, as the majority of ovine ileum exhibits lymphoid rich sites.

2-week period of the study, 66/84 and 56/84 faecal samples were culture-positive for *E. coli* O157:H7 from rectally and orally inoculated lambs, respectively. At the end point of the

study (day 14) four rectally inoculated lambs were shedding *E. coli* O157:H7, whereas only one lamb from the orally inoculated group was shedding *E. coli* O157:H7.

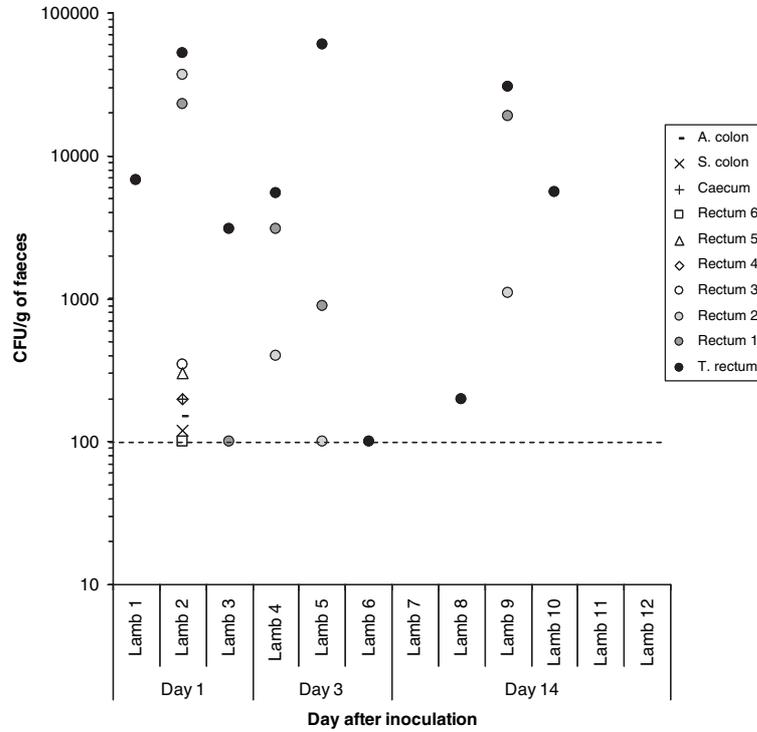


**Figure 3.** The distribution of *E. coli* O157:H7 shedding over time after the inoculation of 6-week-old lambs either (A) rectally or (B) orally with approximately  $1 \times 10^{10}$  CFU. All data points shown are from direct plating only. *E. coli* O157:H7 was not detected from the faeces of lamb 21 during the fourteen day sampling period. Samples where no *E. coli* O157:H7 growth was detected after enrichment are not shown.

**3.4. Recovery of *E. coli* O157:H7 NCTC12900nal<sup>r</sup> from tissues of the gastrointestinal tract of rectally inoculated lambs**

For experiment 2, necropsies were performed after rectal inoculation on three lambs on day 1, three lambs on day 3 and six lambs on day 14 after inoculation, respectively. The

numbers of *E. coli* O157:H7 recovered from different sites along the distal GIT are shown in Figure 4. Recovery of *E. coli* O157:H7 from the faecal samples taken immediately before necropsy is also shown. Only one lamb was culture-positive for *E. coli* O157:H7 for all rectum, spiral and ascending colon, and caecum samples, although numbers were low,



**Figure 4.** Recovery of *E. coli* O157:H7 from the gastrointestinal tract of young lambs rectally inoculated with approximately  $1 \times 10^{10}$  CFU. All data points shown on or above the dotted line are from direct plating only. No *E. coli* O157:H7 growth was detected after enrichment and zero values are not shown.

and that was on day 1. Otherwise, *E. coli* O157:H7 was not recovered from the colon, caecum and proximal rectum samples from any of the remaining 11 animals. No lambs were culture positive for *E. coli* O157:H7 recovery from the duodenum, jejunum and ileum. However, the terminal rectum was positive in 9 of 12 animals examined whereas rectum 1 and rectum 2 samples were positive in 5 of 12 and 4 of 12 animals, respectively.

It was observed that tissues cultured from 3 lambs necropsied had more than  $1 \times 10^4$  *E. coli* O157:H7 CFU/g per terminal rectum shed *E. coli* O157:H7 up to  $10^6$  CFU/g of faeces. By contrast, six lambs with less than  $1 \times 10^4$  *E. coli* O157:H7 CFU/g per terminal rectum only shed lower numbers.

### 3.5. Visualisation of *E. coli* O157:H7 associated with the terminal rectum mucosa

From experiment 2, duodenum, jejunum, ileum, spiral colon, ascending colon, caecum, rectum and terminal rectum tissue sections taken from the gastrointestinal tract were initially fixed in formalin for subsequent staining and analysis by microscopy. Taking into consideration the bacteriological data at necropsy, only tissues from rectum-1 and terminal rectum were selected for staining with anti-O157-FITC for examination by confocal microscopy (Tab. I). Seven of the 12 animals examined exhibited tissues that showed diffuse adherence and small clusters (< 10 bacteria) of *E. coli* O157:H7 to rectum-1 and terminal rectum. Of these, two exhibited large, densely packed micro-colonies at the

**Table I.** Summary of adherence patterns of *E. coli* O157:H7 with the rectum-1 and terminal rectum of rectally inoculated young lambs as observed by confocal microscopy.

Lamb	Necropsy day after <i>E. coli</i> O157:H7 rectal inoculation											
	1				3				14			
	DA		LA		DA		LA		DA		LA	
	R1	TR	R1	TR	R1	TR	R1	TR	R1	TR	R1	TR
1	✓	-	-	-								
2	✓	✓	✓	✓ <sup>+</sup>								
3	✓	✓	-	-								
4					✓	✓	-	✓ <sup>+</sup>				
5					✓	✓	-	✓ <sup>+++</sup>				
6					-	✓	-	-				
7									-	-	-	-
8									-	-	-	-
9									✓	✓	✓ <sup>+</sup>	✓ <sup>+++</sup>
10									-	-	-	-
11									-	-	-	-
12									-	-	-	-

DA, diffuse adherence; LA, localised adherence; R1, rectum-1; TR, terminal rectum; ✓, diffuse adherence pattern observed; ✓<sup>+</sup>, small clusters of *E. coli* O157:H7 observed; ✓<sup>+++</sup>, large densely packed clusters of *E. coli* O157:H7; -, no *E. coli* O157:H7 detected.

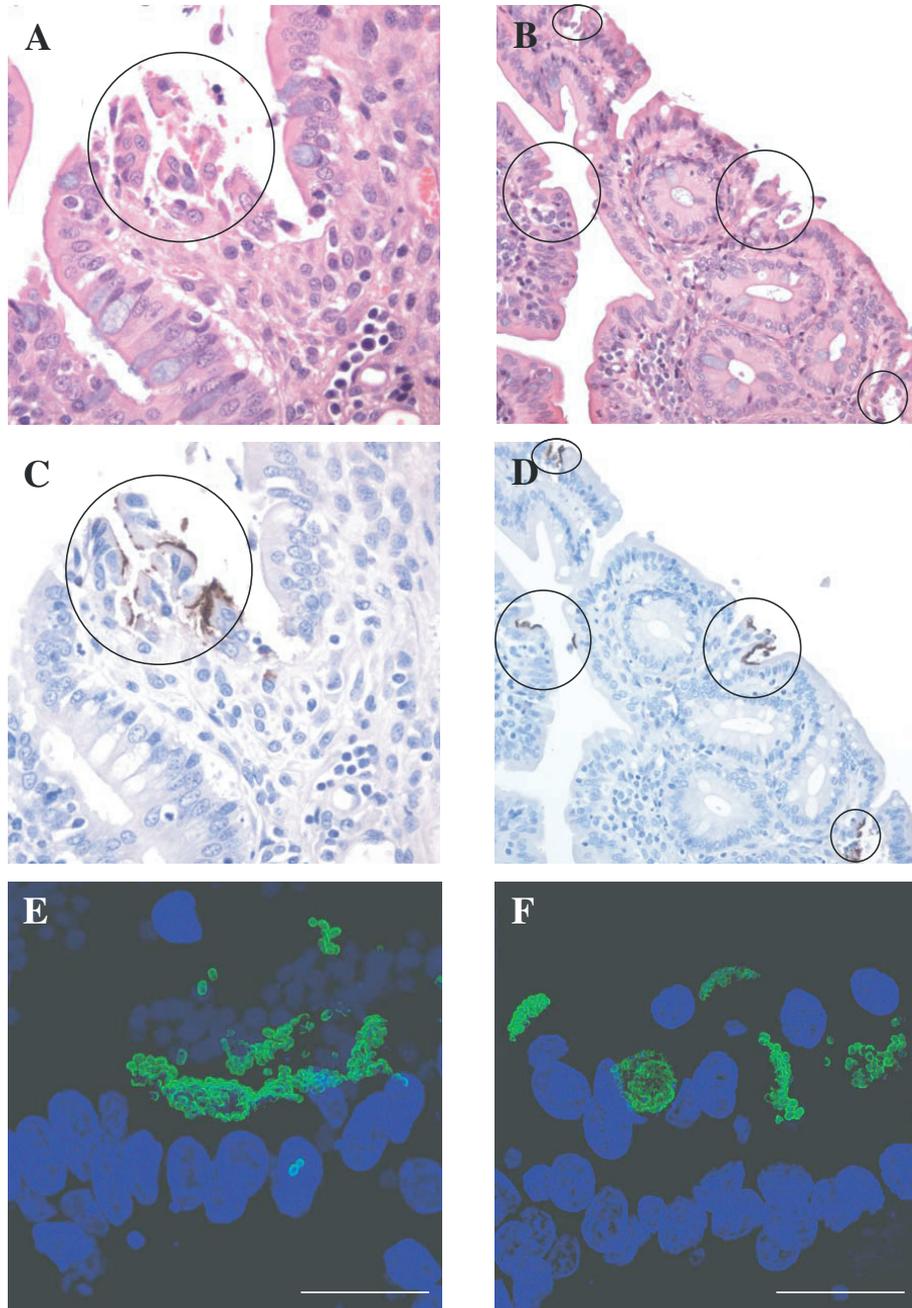
terminal rectum mucosa (Tab. 1 and Fig. 5). By day 14, only 1 of the 6 rectally inoculated animals exhibited large, densely packed micro-colonies colonised at the rectum-1 and terminal rectum sites by *E. coli* O157:H7.

**4. DISCUSSION**

Using previously described IVOC techniques [10, 11] we have demonstrated for the first time that *E. coli* O157:H7 can adhere and associate intimately, albeit sparsely as large, densely packed micro-colonies, on the mucosal surface of explants taken from the terminal rectum of 6-week-old lambs. That the explants are not under the same physiological conditions as in vivo, and the epithelial cell cycle and the metabolism of the explants may have be disrupted, may in part explain why few micro-colonies were observed. Indeed, epithelial cell rate proliferation, which will cease in biopsy, may be linked to the number of *E. coli* O157:H7 micro-colonies formed as indicated by previous cell-proliferation studies [18]. Since micro-colonies were only observed at the mucosa of the ovine terminal rectum, but not the rectum-1 mucosa, this

may suggest a tissue tropism for this phenotype. However, the numbers of samples evaluated for the present study were limited and care should be taken in drawing firm conclusions. Nevertheless, a previous bovine IVOC study did demonstrate the development of micro-colonies at the bovine terminal rectum mucosa, although foci of intimately attached *E. coli* O157:H7 were observed at the ileum and colon [11].

Oral inoculation studies in sheep have suggested that the lower GIT is the primary location for *E. coli* O157:H7 colonisation [12, 28]. Since the *E. coli* O157:H7 used presently was Stx-negative, we undertook a detailed bacterial enumeration of ovine tissues from orally challenged lambs with a focus towards the terminal rectum and showed that on days 5 and 7 after inoculation, greater numbers of *E. coli* O157:H7 were recovered from the terminal rectum, as compared to other tissues of the GIT. Unlike Naylor et al. [22] for cattle, there was no conclusive evidence from our bacterial enumeration data, that *E. coli* O157:H7 preferentially associated with lymphoid follicles at the terminal rectum in sheep. Indeed, in some lambs



**Figure 5.** Light and confocal microscopy of *E. coli* O157:H7 localised adherence and micro-colony formation at the terminal rectum mucosa of rectally inoculated young lambs at day 3 (Lamb 5: A, C and E) and 14 (Lamb 9: B, D and F) after inoculation. Open circles denote localised adherence. Staining: (A and B) = Haematoxylin and Eosin; (C and D) = Meyer's haematoxylin (terminal rectum-blue), immunoperoxidase (*E. coli* O157-brown); (E and F) Dapi (blue) = nuclei; FITC (green) = *E. coli* O157. Magnification (A and C) = 400 $\times$ , (B and D) = 200 $\times$ . Bar (E) = 20  $\mu$ m, (F) = 18  $\mu$ m.

more *E. coli* O157:H7 were associated with non-lymphoid tissue, suggesting no clear-cut tropism for lymphoid follicles. Also, analysis of immune cell infiltration did not reveal any heightened response to the presence of *E. coli* O157:H7 at the terminal rectum. In contrast, cattle studies have reported a local IgA response to *E. coli* O157:H7 colonisation of the terminal rectum [16, 21]. However, this local response did not clear *E. coli* O157:H7 from this primary colonisation site in cattle. Previous studies have suggested that during the first seven days after oral inoculation of sheep, *E. coli* O157:H7 may be passing through the digesta rather than colonising specific regions of the GIT [12, 26]. Nevertheless, the necropsy data presented here does indicate that on day 5 and 7, but not on day 3 after inoculation, *E. coli* O157:H7 did exhibit a tissue tropism towards the distal GIT. Therefore, a modified rectal administration methodology was initiated to test this hypothesis.

Rectal administration of *E. coli* O157:H7 using a cylindrical sponge previously demonstrated enhanced long-term persistence in cattle [26]. Since oral inoculation involves dispensing the inoculum into the pharynx, we wished to administer the inoculum in a similar manner onto the distal end of the rectum, so that the only experimental variable was the passing of *E. coli* O157:H7 through the GIT. At necropsy of *E. coli* O157:H7 rectally inoculated lambs, the terminal rectum was colonised with *E. coli* O157:H7 most frequently, and in some lambs was the only associated tissue at days 1, 3 and 14 after rectal inoculation. Rectum-1 was colonised with *E. coli* O157:H7, but in fewer lambs even though the inoculum was delivered proximal to this site. Nevertheless, confocal microscopy revealed that not all lambs that were *E. coli* O157:H7 culture-positive for terminal rectum, demonstrated *E. coli* O157:H7 micro-colonies on the terminal rectum mucosa. At the end point of this study, only one lamb which generally shed *E. coli* O157:H7 in higher numbers than all other lambs, exhibited large, densely packed micro-colonies. That five other remaining lambs at this time point did not demonstrate

*E. coli* O157:H7 micro-colony formation with the terminal rectum, does suggest that within this cohort of animals, preferential colonisation of this site did not occur. Furthermore, if indeed the terminal rectum was the primary *E. coli* O157:H7 site, we may have noted rapid colonisation of this site as reported for cattle [5, 17, 26]. To further support the necropsy data, no significant differences in *E. coli* O157:H7 faecal shedding of 6 rectally and 6 orally inoculated lambs were noted from day 2 to day 14 after inoculation. That greater numbers were recovered from the faeces of rectally inoculated lambs on day 1 after inoculation is an unsurprising finding given the oral inoculum has to travel through the GIT before being shed.

Previous oral inoculation sheep studies from other laboratories have used mature, fully ruminating sheep to monitor *E. coli* O157:H7 persistent colonisation. Grauke et al. [12] did isolate the highest numbers of *E. coli* O157:H7 from the lower GIT, but the recovery of positive cultures was inconsistent and only occurred during the first week after oral inoculation. However, after an abrupt dietary change 42 days after inoculation, *E. coli* O157:H7 was recovered from the rectal tissue of one animal. A subsequent rectal administration model of *E. coli* O157:H7 colonisation in adult ruminants did further support the findings of Naylor et al. [22] that the terminal rectum mucosa is the primary colonisation site in cattle [26]. Interestingly, the authors of the latter study discuss *E. coli* O157:H7 oral and rectal challenge of fully mature cattle, but only the oral challenge of fully mature sheep. Whether the authors did rectally swab fully mature sheep with *E. coli* O157:H7 and a poor take was observed was not discussed. Importantly, the rectal administration method used presently involved applying a flush of *E. coli* O157:H7 in liquid rather than inserting a saturated sponge swab. As discussed previously [26], by its' very nature, rubbing a saturated sponge against the terminal rectum mucosa will have contributed to the enhanced colonisation of *E. coli* O157:H7 and in part may explain why consistent colonisation of the ovine terminal

rectum was not observed. Nevertheless, the literature does suggest that locating the primary *E. coli* O157:H7 colonisation site in adult sheep is thwarted with difficulties.

Intermittent *E. coli* O157:H7 colonisation of the terminal rectum may be in part explained by the differences in the mechanics of ovine and bovine faeces formation. Despite ovine and bovine species having physiological similarities with respect to the spiral colon, in the ovine host this region of the GIT is the only site of faecal pellet formation. In sheep, this distinctiveness is coupled with a permanent and localised contractile activity to give faeces in the form of pellets, whereas in cattle, faeces are formed during 5 min phases of continuous slow migrating activity away from the proximal GIT (aborally) [25]. These mechanical and physiological differences between the faecal types (hard versus soft) may imply that *E. coli* O157:H7 in the digesta of the GIT, become encased within the hard faecal pellets at the ovine spiral colon, thus reducing the chances of *E. coli* O157:H7 colonisation at the terminal rectum mucosa.

Whether Shiga-toxin plays a significant role in colonisation of the terminal rectum remains unclear, although Naylor et al. [22] did demonstrate that a naturally occurring Stx-negative human outbreak *E. coli* O157:H7 strain did preferentially colonise the terminal rectum mucosa. Furthermore, Cornick et al. [8] has reported that Shiga-toxin does not facilitate *E. coli* O157:H7 colonisation in sheep.

In conclusion, the studies presented here are the first to report that *E. coli* O157:H7 can form micro-colonies on ovine terminal rectum explants and colonise the terminal rectum of rectally inoculated young lambs. Although, the in vivo data does suggest that the ovine terminal rectum may be colonised by *E. coli* O157:H7, colonisation is less efficient than in cattle. Furthermore, and in contrast to rectally inoculated cattle [5, 17, 26] the numbers of *E. coli* O157:H7 shed in ovine faeces over the time-course of the study were not significantly different to those of orally inoculated lambs. We suggest that a more comprehensive understanding of the

factors that promote effective colonisation at the ovine terminal rectum requires further analysis in order to assess the appropriateness of intervention strategies [4, 19, 20] for use in controlling *E. coli* O157:H7 in the field.

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