Comparative Medicine
Copyright 2010
by the American Association for Laboratory Animal Science

Vol 60, No 6
December 2010
Pages 469–478

Catheterization of Intestinal Loops in Ruminants Does Not Adversely Affect Loop Function

G Douglas Inglis,[1,*] John P Kastelic,[1] and Richard R E Uwiera[2]

Catheterized intestinal loops may be a valuable model to elucidate key components of the host response to various treatments within the small intestine of ruminants. We examined whether catheterizing ileal loops in sheep affected the overall health of animals and intestinal function, whether a bacterial treatment could be introduced into the loops through the catheters, and whether broad-spectrum antibiotics could sterilize the loops. *Escherichia coli* cells transformed to express the GFP gene were introduced readily into the loops through the catheters, and GFP *E. coli* cells were localized within the injected loops. Catheterized loops, interspaces, and intact ileum exhibited no abnormalities in tissue appearance or electrical resistance. Expression of the IFNγ, IL1α, IL4, IL6, IL12p40, IL18, TGFβ1, and TNFα cytokine genes did not differ significantly among the intact ileum, catheterized loops, and interspaces, nor did the expression of the gene for inducible nitric oxide synthase. Broad-spectrum antibiotics administered during surgery did not sterilize the loops or interspaces and did not substantively change the composition of the microbiota. However, antibiotics reduced the overall number of bacterial cells within the loop and the relative abundance of community constituents. We concluded that catheterization of intestinal loops did not adversely affect health or loop function in sheep. Furthermore, allowing animals to recover fully from surgery and to clear pharmaceuticals will remove any confounding effects due to these factors, making catheterized intestinal loops a feasible model for studying host responses in ruminants.

Abbreviations: EMA, ethidium monoazide; GFP, green fluorescent protein; iNOS, inducible nitric oxide synthase; T-RFLP, terminal restriction fragment length polymorphism; T-RF, terminal restriction fragment; TGFβ1, transforming growth factor β1.

The intestine is a complex organ that not only absorbs nutrients but also acts as a barrier between the individual and the outside world. The intestine plays a pivotal role in immunosurveillance and protection from enteric pathogens. Investigations of intestinal physiologic and immunologic function use several experimental models, including xenografts and intestinal loops. Xenograft intestinal models involve fetal intestinal segments that have been harvested from a donor of one species and transplanted into an immunodeficient member of another species.[34] These xenografted intestinal segments develop into functional adult-like 'microorgans,'[5] facilitating assessment of localized mucosal responses to various treatments including pathogens.[17,32] Intestinal loops are an alternative model to xenografts. Many loop models have been nonrecovery surgical procedures that measured short-term (less than 24 h) changes in the intestine.[31,38] We previously created a recovery intestinal loop model specifically to measure long-term (> 6 mo) immunologic changes in the intestine of sheep after exposure to vaccines, adjuvants, and viruses.[16] In contrast to xenografts and nonrecovery loop models, recovery loop models enable measurement of systemic immunologic responses within the small intestine (for example, trafficking of lymphocytes and other immunocompetent cells). Salient limitations of recovery loop models include a single window of opportunity to administer agents to loops (at the time of surgery), the confounding effects of therapeutic agents (for example, antibiotics, antiinflammatories, analgesics) on the study of bacterial treatments or host responses, and samples of intestinal mucosa and luminal contents can only be harvested at the termination of the project. Therefore, we modified our recovery loop model by inserting long-term catheters into the intestine.[36] Validation of this model requires determination of whether the catheterization procedure adversely affects animal health, whether catheterization alters loop function, whether treatments can be deposited into loops through the catheters after recovery from surgery, and whether administration of broad-spectrum antibiotics creates axenic (that is, devoid of microorganisms) conditions within the loops as previously concluded.[1,2,16,26] Therefore, the objectives of the current study were to determine the: 1) effect of catheterization on animal health, 2) deposition and localization of green fluorescent protein (GFP) *Escherichia coli* in loops, 3) effect of catheters on the physiologic and functional responses of loop tissues, and 4) effect of broad-spectrum antibiotics on the intestinal microbiota within loops.

Received: 26 May 2010. Revision requested: 04 Jul 2010. Accepted: 29 Jul 2010.
[1]Agriculture and Agri-Food Canada, Lethbridge, and [2]Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada.
[*]Corresponding author. Email: Douglas.Inglis@agr.gc.ca

Materials and Methods

Animals. Canadian Arcott sheep (female; age, 8 to 10 mo; n =5) from a closed flock were used; individual animals were treated as replicates. All surgeries and necropsies were conducted at the Agriculture and Agri-Food Canada Research Centre at Lethbridge (Canada). Approval by the IACUC in accordance with the standards specified by the Canadian Council on Animal Care[8] was obtained before beginning the study (LRC Animal Use Protocol 0609). Approval to use genetically modified *Escherichia coli* was obtained from the Biosafety and Biosecurity Committee of

the Agriculture and Agri-Food Canada Research Centre at Lethbridge. Sheep were maintained on a diet of alfalfa–grass hay and (except for the immediate postoperative period) were allowed ad libitum access to feed and water.

Surgical procedure. A detailed description of the surgical procedure has been published.[36] Sheep were premedicated with acepromazine (0.05 mg/kg IM), glycopyrrolate (0.005 mg/kg IM), and butorphanol tartrate (0.2 mg/kg IM). Approximately 20 min later, diazepam (0.2 mg/kg IV) was administered, and anesthesia was induced with thiopental sodium (10 mg/kg). Sheep were intubated and maintained on isoflurane (1.5% to 2.5% in 100% O_2 at a rate of 4 L/min) for the duration of the surgery. Sheep were placed in dorsal recumbency, a 15-cm midline abdominal incision was made, and the ileocecal fold was located. The caudal ileum and cecum were exteriorized. Approximately 85 cm of functional ileum (that is, possessing continuous Peyer patches) was clamped with intestinal clamps and large Crile forceps at each end of the segment. The intestinal clamps were placed on the side of the ileum to be rejoined (that is, the anastomosis), whereas the Crile forceps were placed on the intestinal segment side of the ileum. The ileum was cut between each pair of intestinal and Crile forceps, and the intestinal segment designated for loops was flushed twice with 60 mL warm PBS to remove ingesta. A cocktail of broad-spectrum antibiotics (200 mg enrofloxacin; 250 mg metronidazole; 60 mL total volume) was distributed throughout the intestinal segment and left for 30 min. The nonintestinal segment side of the ileum was rejoined to form a continuous and functioning intestinal tract. The ileum was aligned by 2 stay sutures at the mesenteric and antimesenteric borders of the intestine, a simple continuous suture pattern (2-0 Vicryl; Ethicon, Johnson and Johnson, New Brunswick, NJ) was completed on both sides of the intestine, and the integrity of the anastomosis was confirmed. The antibiotic cocktail was removed from the intestinal segment, and each end of the segment was closed with a simple continuous suture pattern (absorbable suture; 2-0 Vicryl, Ethicon) followed by an inverting suture pattern of 2-0 Vicryl (Ethicon).

The intestinal segment was partitioned into three 15-cm 'loops', two 15-cm 'interspaces' between the loops, and two 5-cm blunt-end compartments at the termini of the intestinal segment by ligatures of 2-0 silk. Catheters (silastic tubing; inside diameter, 1.0 mm; outside diameter, 2.1 mm) were identified individually by using a permanent marker. A silicon ball (approximately 3 to 4 mm in diameter) was placed 3 to 4 cm from the catheter end to help prevent the catheter from sliding out of the intestine. After sterilization by autoclaving, a nonantibiotic ointment was inserted aseptically into the catheter end (3 to 5 mm) to ensure that the catheter remained patent within the lumen of the loop. To establish the catheter in each loop, a small incision was placed at the cranial and antimesenteric aspect of the loops, and an approximate 4- to 5-cm segment of catheter was inserted caudally into the loop until the silcone ball passed into the lumen. The intestinal wall was secured around the catheter with a pursestring suture of 2-0 Vicryl (Ethicon), and the catheter was secured to the intestine further by tying the catheter with the ligature silk that delineated the cranial end of the loop. Ampicillin was injected into each loop and interspace (total of 500 mg in 5 mL warm PBS), and the intestine was reintroduced into the abdominal cavity. Cefazolin (8 mL; 100 mg/mL) in warm PBS was injected into the peritoneal cavity through the abdominal incision. A single small stab incision was made through the abdominal wall adjacent to the midline incision, and the catheters were exteriorized through this site. Abdominal muscles were closed with a simple interrupted suture pattern (absorbable suture; catgut no. 3), and the skin was closed with an interrupted horizontal mattress pattern and (nonabsorbable suture; Supramid no. 1, Ethicon).

A curved hollow stainless-steel tube (inner diameter, 7 mm; outer diameter, 10 mm; length, 65 cm) was inserted subcutaneously near the abdominal incision and tunneled to an exit site just caudal to the neck and between the shoulders. The catheters were inserted into the tube and pushed forward more than half of the tube length; the tube with the catheters then was pulled from the skin at the exit site, thereby exposing the catheters. The tension of the catheters was adjusted at the stab incision site, and the skin was closed with Supramid no. 1 (Ethicon). Individual catheters were identified and placed within a bandage pouch, which was sutured to the skin by using Supramid no. 1 (Ethicon). The duration of the surgical procedure ranged from 105 to 120 min.

Postoperative care. Under anesthesia, sheep were injected with flunixin at an initial dose of 2.2 mg/kg IM, followed by 1.1 mg/kg IM once daily for 3 d. In addition, sheep were injected intramuscularly with a B-vitamin mixture (3 mL; Hemostam, Rafter 8 Products, Calgary, Canada) and enrofloxacin (2.5 mg/kg) once daily for 3 and 5 d, respectively. Animals were maintained on intravenous Plasmalyte 148 with 5% dextrose (Baxter Healthcare, Deerfield, IL) solution. They were allowed to drink water ad libitum, but feed was restricted until normal rumen and bowel functions were restored. Food intake, water consumption, body temperature, passage of feces and urine, gut noises, abdominal discomfort, demeanor, and blood glucose concentrations were closely monitored twice daily. When necessary, additional dextrose was added to the intravenous fluids to maintain plasma blood glucose concentrations within the physiologic range. Blood chemistry parameters (that is, albumin, alkaline phosphatase, alanine aminotransferase, amylase, calcium, cholesterol, creatinine, glucose, inorganic phosphate, total bilirubin, total protein, urea, and globulin), electrolytes and blood gases (that is, sodium, potassium, chloride, partial pressure oxygen, pH, partial pressure carbon dioxide, bicarbonate, and total carbon dioxide), and CBC parameters (that is, RBC and WBC counts, hematocrit, hemoglobin, differential WBC counts, and platelet counts) were monitored before and immediately after surgery and alternate days thereafter.

Once normal bowel function commenced, intravenous fluid therapy was terminated, and sheep were transferred to paddocks. Sheep were maintained in individual pens within sight of other sheep to reduce stress and the chance of sustaining damage to their catheter pouches (for example, by chewing). During this period, sheep were observed daily for any evidence of distress, fecal output, and feed and water intake. In addition, sheep received a weekly health assessment. The examination included assessment of demeanor, respiration rate, temperature, and examination of the catheter exit and abdominal incision sites for evidence of infection (for example, occurrence of pain or tenderness, localized swelling, redness, or heat and evidence of an abscess or purulent drainage). At 3 to 4 d before tissue harvest, animals were transferred back to the surgical facility, placed in crates, and allowed to feed and drink water ad libitum.

Inoculation of loops. To examine the integrity of the catheterized loops, we arbitrarily selected a single loop per sheep and inoculated the loop with *E. coli*. *E. coli* (DH5α) was transformed

with the plasmid pWM1007, which contains a consensus *Campylobacter jejuni* promoter and the GFP gene.[11] Transformed cells were grown in Luria broth with kanamycin (100 µg/mL) for 16 h at 37 °C with shaking at 100 rotations per minute. To harvest GFP *E. coli* cells, the cell suspension was centrifuged at 14,900 × *g* for 10 min, the supernatant was removed, cells in the pellet were resuspended in PBS, and cell density was adjusted to an OD_{600} of 0.3. This turbidity corresponded to a cell density of approximately 10^7 cfu/mL as determined by dilution plating of the suspension on MacConkey agar. GFP *E. coli* cells were diluted 10-fold with PBS to bring the concentration to 10^6 cfu/mL. PBS alone or containing GFP *E. coli* cells (5 mL) were placed into 10-mL syringes each fitted with a sterile 18-gauge blunt-ended needle. Approximately 1 h before induction of anesthesia (that is, for tissue harvest), catheters were removed from the pouch, and all catheters were injected with 3 mL PBS to ensure patency. A single catheter was arbitrarily selected per sheep, and into this catheter GFP *E. coli* cells were injected (5 mL), followed by injection of 3 mL PBS to ensure that all cells were deposited in the loop lumen. The remaining 2 loops per animal were similarly injected with 5 mL and then 3 mL PBS.

Tissue harvest. Animals were anesthetized as described previously.[36] Under anesthesia, a paramedian incision approximately 3 to 5 cm from the midline was made, and the ileum and intestinal segment were exteriorized. To determine whether the GFP *E. coli* leaked into the peritoneum from injected loops, 5 swabs were obtained from random locations within the peritoneal cavity. Viscera was examined for evidence of pathologic changes (for example, peritonitis), and the integrity of the anastomosis site was evaluated visually. From each animal, the 3 catheterized loops, the 2 interspaces between the catheterized loops, and a segment of intact ileum proximal and distal to the anastomosis site were aseptically harvested. Each tissue segment was processed completely before the next one was harvested. Care was taken to minimize blood loss during the tissue collection procedure. The excised intestinal tissue was immediately placed on ice. A small incision was made in the intestinal wall of all loops and interspaces, the intestinal contents were collected by gravity, and the contents placed on ice.

From the 2 nonGFP *E. coli*-inoculated loops, the 2 interspaces, and the 2 intact ileal samples, a segment of the intestine (approximately 5 cm long) was removed quickly for Ussing chamber analysis. Immediately, each segment was placed in carboxygenated Krebs bicarbonate buffer[35] for measurements of electrical resistance. The remaining tissue of each segment was longitudinally incised, the surface of the mucosa was washed gently with sterile chilled PBS (taking care to minimize disruption of mucus), and the mucosal surface was examined for visible abnormalities and photographed. Within approximately 3 to 5 min of removal from the sheep, 3 sections (1 cm^2) of intestine (arbitrarily selected locations) were removed aseptically and placed in RNAlater (Ambion, Applied Biosystems Canada, Streetsville, Canada) for subsequent RNA extraction. Another 1-cm^2 section was removed and placed in a sterile tube on ice for subsequent microbiologic analysis. Biopsy plugs (diameter, 3 mm) were removed aseptically and frozen (−20 °C) for DNA extraction, and multiple sections (approximately 0.5 × 0.5 cm) of tissue were placed in cassettes in freshly prepared 10% buffered neutral formalin for subsequent histologic examination. Once tissue collection was complete, the sheep was euthanized under anesthesia by intravenous injection with sodium pentobarbital (approximately 2.0 mL/kg; Bimeda-MTC Animal Health, Cambridge, Canada).

Detection and quantification of GFP *E. coli*. Swabs and the luminal contents (1 mL) and mucosal surfaces (1 cm^2) of all loops and interspaces were processed for GFP *E. coli.* Mucosal samples were homogenized in 1 mL Bolton broth (Oxoid, Nepean, Canada) for 30 s by using a tissue homogenizer (Fisher Scientific, Edmonton, Canada). The mucosal homogenate and luminal samples were diluted in Bolton broth in a 10-fold dilution series, 100 µL was spread onto MacConkey Agar containing 100 µg/mL kanamycin, cultures were maintained at 37 °C, and colonies were enumerated at a dilution yielding 30 to 300 cfu. Swabs from the peritoneal cavity were agitated in 2 mL of Luria broth, the broth was streaked onto MacConkey Agar containing kanamycin, and cultures were maintained at 37 °C. Representative colonies were streaked onto fresh MacConkey agar containing kanamycin, cultures were maintained at 37 °C for 48 h, and colonies were examined for the release of green pigment into the medium. DNA was extracted from bacterial cells robotically (AutoGen, Farmingham, MA), and the presence of the GFP-containing plasmid was determined by PCR using GFP plasmid-specific primers developed in the present study. Sequence information for the GFP plasmid was taken from GenBank (accession no. AF292556.1), and the primers Gfp825f (5′ GGT ATA ATT AGA ATT CGG CTT A 3′) and Gfp1409r (5′ GTC TGC TAG TTG AAC GCT TCC A 3′) were developed by using Oligo (Molecular Biology Insights, Cascade, CO). Samples were amplified as follows: initial denaturation at 95 °C for 15 min; 35 cycles of 30 s at 94 °C, 60 s at 50 °C, and 60 s at 72 °C; and extension for 10 min at 72 °C. The PCR mixture consisted of a total volume of 20 µL containing 1× reaction buffer, 0.2 mmol/L dNTP, 2 mmol/L $MgCl_2$, 0.5 µmol/L each primer (Sigma-Genosys, Oakville, Canada), 0.2 µg bovine serum albumin (Promega, Madison, WI), and 0.5 U HotStar *Taq* polymerase (Qiagen, Mississauga, Canada). Each PCR reaction was performed with a total of 2 µL DNA that had been diluted 100× with purified water. PCR products (10 µL) were electrophoresed on a gel containing 1% agarose (Invitrogen, Burlington, Canada) in Tris acetate–EDTA buffer and visualized by staining with ethidium bromide. A 100-bp ladder (New England Biolabs, Ipswich, MA) was used to determine product size. The GFP amplicon was 584 bp.

Direct detection of GFP *E. coli* also was conducted. DNA was extracted from luminal contents and mucosal tissues of all loops and interspaces by using the DNeasy Blood and Tissue Kit (Qiagen). DNA then underwent PCR amplification of the GFP plasmid as just described for isolates, except that 2 µL undiluted template was used in the PCR mixture.

Histopathology. Fixed intestinal samples were processed for hematoxylin and eosin staining according to standard protocols. Tissue sections were assessed by a veterinary pathologist (Richard Uwiera) who was 'blinded' to the type of intestinal segment submitted (that is, catheterized loops, interspaces, and intact ileum). These tissues were examined for mucosal necrosis and villous blunting as well as indicators of intestinal inflammation, tissue congestion, tissue fibrosis, and lympholysis. Three representative fields of views at magnification ×100 were examined per slide and treated as observations. Histologic sections were scored as: 0, normal; 1, minor; 2, moderate; and 3, marked change.

Cytokine and iNOS gene expression. Primers used to quantify ovine cytokine and iNOS gene expression are shown in Table 1.

Table 1. RT-PCR primers for cytokine, iNOS, and housekeeping genes.

Gene	Primer	Sequence (5′-3′)	Amplicon (bp)	Reference
IFNγ	obIFNgF	GTG GGC CTC TTT TCT CAG AA	234	This study
	bIFNgR	GAT CAT CCA CCG GAA TTT GA		33
IL1α	bIL1aF	GAT GAT GAC CTG GAA GCC ATT	209	33
	bIL1aR	TTT CAC TGC CTC CTC CAG AT		33
IL1β	bIL1bF	AAA TGA GCC GAG AAG TGG TGT T	185	33
	bIL1bR	TTC CAT ATT CCT CTT GGG GTA GA		33
IL4	bIL4F	AGA GAT CAT CAA AAC GCT GAA CAT	195	37
	oIL4R	TCC TGT AGA TAC GCC TAA GAC TCA A		This study
IL6	bIL6F	TCA GCT TAT TTT CTG CCA GT	105	3
	bIL6R	TCA TTA AGC ACA TCG TCG ACA AA		3
IL12 p40	bIL12p40F	ATT GAG GTC GTG GTA GAA GCT G	112	3
	bIL12p40R	GGT CTC AGT TGC AGG TTC TTG G		3
IL18	oIL18F	TCA GCT CTC CTG GCG GTC T	123	This study
	oIL18R	ATG CCT GTG CTC AAT AGC TTC C		This study
TNFα	oTNFaF	AGC TGG CCC CTC CTT CAA CA	103	This study
	bTNFaR	CCA TGA GGG CAT TGG CAT AC		3
TGFβ1	bTGFb1F	CTG AGC CAG AGG CGG ACT AC	132	13
	bTGFb1R	GGA GCT CGG ACG TGT TGA A		This study
iNOS	bINOSF	ACC TAC CAG CTG ACG GGA GAT	195	29
	oINOSR	CGA CCT GAT GTT GCC GTT GTT		This study
GAPDH	bGAPDHF	CCT GGA GAA ACC TGC CAA GT	226	33
	bGAPDHR	AAT TCA TTG TCG TAC CA		33
β2 microglobulin	oB2MF	TAT ACT CAA GAC ACC CGC CAG A	176	This study
	oB2MR	AGC GTG GGA CAG AAG GTA GAA		This study

Primers derived from bovine sequence are indicated with 'b'; those from ovine sequence are indicated with 'o'.
The annealing temperature for all primers was 54 °C.

Where primers were not available for ovine cytokines, iNOS, or housekeeping genes, ovine or bovine sequence data for target genes was obtained from GenBank, and primers were developed by using Oligo (Molecular Biology Insights). All developed primer sets were tested to verify that amplification was linear and repeatable. In all instances, coefficients of determination were greater than 0.97, and efficiencies were between 95% and 105%. The DNA used for validations was derived from reverse-transcribed RNA isolated from mucosal tissue.

RNA was extracted from intestinal tissues by using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol for isolation of total RNA from animal tissues. Reverse transcription was conducted by using the QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's protocol. Reverse transcription–quantitative PCR was conducted using the QuantiTect SYBR Green PCR kit in an Mx3005P thermocycler (Stratagene Products, La Jolla, CA). For each sheep, all tissues and genes were processed simultaneously in duplicate. Each plate contained a control sample (calibrator sample) and a housekeeper gene (GAPDH). Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.[39] RNA extracted from mucosal tissue of a catheterized loop injected with PBS was used as the control. The mean of the 2 duplicate samples was calculated, and data were analyzed by using the MIXED procedure of the SAS software (SAS Institute, Cary, NC). Sheep and the sheep × tissue interaction were treated as random effects, and tissue was treated as a repeated measure. The appropriate covariance structure was used according to the lowest Akaike Information Criterion (SAS Institute).

Tissue electrical resistance. Intestinal tissues in Krebs bicarbonate buffer were transferred to the Ussing chamber (Navicyte Vertical Diffusion Chamber, Harvard Apparatus Canada, Saint-Laurent, Canada) laboratory within 2 to 3 min of tissue removal. Segments were opened along the mesenteric border, gently rinsed free of intestinal contents, cut into rectangles (2 × 3 cm), and mounted between the halves of the Ussing chamber (Navicyte Vertical Diffusion Chamber, Harvard Apparatus Canada) with an exposed area of 1.92 cm^2. Care was taken to ensure that Peyer patches were not placed within the analysis area. A maximum of 15 min elapsed from tissue harvest to commencement of incubation. Tissues were bathed on both sides with Krebs buffer[35] (7 mL per side) at 37 °C and equilibrated for 10 min before commencement of electrical measurements. The buffer solution was mixed and gassed with carbogen (95% O_2, 5% CO_2) by using a gas lift system. Electrical measurements were obtained (VCC MC6 Voltage–Current Clamp, Physiologic Instruments, Harvard Apparatus Canada). After the equilibration period, the potential difference and short-circuit current were recorded every 20 min for 180 min. Tissue resistance ($\Omega\ cm^2$) was calculated by using the Ohm law from the open-circuit potential difference and short-circuit current.[7,25] Data were analyzed with the MIXED procedure of SAS (SAS Institute), with tissue type, time, and their interaction treated as fixed effects. Tissue and time were treated as repeated measures, and the appropriate covariance structure was used according to the lowest AIC. Contrast statements were used to make comparisons among tissue types (that is, across subsamples).

Microbiota. To each mucosal sample (diameter of biopsy, 3 mm), 200 µL 1:10 dilution of AE buffer (Qiagen) was added. To identify bacterial cells with intact cell membranes, 4 µL of a solution of ethidium monoazide (EMA) in water (100 µg/mL) was added to half of the mucosal samples within 2 h after removal from the sheep.[21] Water (4 µL) was added to the remaining non-EMA-treated samples. All samples were incubated on ice for 5 min, the tubes lids opened, and the tubes contents were exposed to light generated from a 500-W halogen lightbulb for 1 min. The light source was situated 10 cm above the tubes. Tubes were stored at −20 °C until DNA was extracted by using the Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol for gram-positive bacteria (this protocol extracts DNA from both gram-positive and gram-negative bacteria).

Eubacteria were quantified by quantitative PCR of the 16S rRNA gene using an Mx3005P thermocycler (Stratagene) as described previously.[9] DNA extracted from EMA-treated mucosal samples was used. A standard curve was established with genomic DNA from *E. coli* (ATCC 25922). Because *E. coli* ATCC 25922 contains 7 copies of the 16S rRNA gene[10] and because the fragment amplified was 150 bp in size, the number of copies of the 16S rRNA gene in 100 ng genomic DNA was calculated as:

$$(\{100\ ng\ /\ [(4.646 \times 10^6\ bp \times 649\ g/mol/bp) \times 10^9\ ng/g]\} \times 6.02 \times 10^{23}\ molecules/mol) \times 7$$
$$= 1.40 \times 10^8\ copies.$$

For all reactions, melting curve analysis was conducted to confirm amplification specificity. Data were analyzed by using the MIXED procedure of SAS (SAS Institute) as described for analysis of cytokines. When the F test was significant, the least square means statement with the probability of difference option was used to identify differences among treatments. The average of the 2 subsamples per tissue type was examined.

To examine the community structure of living bacteria associated with the mucosa of the loops, interspaces, and intact ileum, terminal restriction fragment length polymorphism (T-RFLP) analysis[12] was applied to DNA exposed to EMA. Terminal restriction fragment (T-RF) were separated by using a model 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) with a Genescan LIZ600 size standard (Applied Biosystems). Scans were exported and peaks sized by using Genemapper 4.0 software (Applied Biosystems). Data were exported to T-REX,[14] true peaks were identified (2 SD), and data matrices were constructed. Matrices for both presence and absence of T-RF and relative abundance of T-RF (that is, based on peak heights) were generated. Data were imported into Bionumerics software version 5.10 (Applied Maths, Austin, TX) for cluster and genetic similarity analyses. Cluster analysis was performed by using the Dice coefficient, and unweighted pair–group methods with arithmetic means (UPGMA). To test the statistical significance ($P < 0.05$) of differences for each tissue type, multivariate analysis was performed and the within- and between-group similarities were compared.[12] To assess community structures, the nonmetric multidimensional scaling procedure in SAS (SAS Institute) was performed on a similarity matrix generated by using the Bray–Curtis distance measure on relative abundance T-RFLP data. This protocol is an ordination technique that arranges samples in multidimensional space on arbitrary axes according to their similarity or dissimilarity, such that samples that are highly similar appear close together, whereas less similar samples appear further apart. To construct the Venn diagram, T-RF associated with the 3 tissue types (combined across replicate and subsample) were calculated from the information obtained from T-REX.

Results

Postoperative care. Ileus occurred in all sheep after surgery, lasting for as long as 7 d. Our previous experience demonstrated that uncontrolled ingestion of feed, particularly alfalfa hay, after recovery from anesthesia resulted in impaction of the rumen and loss of rumen function in sheep. Therefore feed after surgery was restricted to 200 to 500 g grass hay (depending on appetite) per sheep daily until normal bowel function was restored. In addition, we found that maintaining sheep on intravenous dextrose was an essential component of postoperative care to prevent hypoglycemia. Ensuring blood glucose concentrations of 0.5 to 0.8 g/L was critical during the period of ileus. Body temperatures of all sheep remained within the normal range throughout the postoperative period, and sheep did not exhibit evidence of overt discomfort or distress. Bowel function was restored 3 to 7 d after surgery. There was no indication of infection in any sheep. During the 34 to 48 d after surgery that sheep remained in the paddocks, there was no evidence that the presence of catheterized loops adversely affected the sheep, in that appetite, thirst, demeanor, socialization, movement, temperature, respiration, and fecal output remained normal.

Inoculation of loops. All catheters were patent, enabling injection of saline. However, previous experience indicated that care must be taken to ensure the catheters do not become restricted by excessively tightening the ligation suture attaching the catheters to the loop, thereby obstructing passage of fluid through the catheter. The GFP *E. coli* that we injected into one loop per sheep approximately 1 h before induction of anesthesia for tissue harvest remained localized within the inoculated loops. In no instance

was GFP *E. coli* isolated from the peritoneal cavity (that is, from the 5 swabs taken from the peritoneal cavity of each sheep) or interspaces adjacent to the injected catheterized loop. Furthermore, none of the interspaces (luminal contents or mucosa) adjacent to inoculated loops were PCR-positive for the GFP gene. Substantive numbers of GFP *E. coli* (average, 3.53 ± 0.27 $\log_{10}$ cfu/mL) or plasmids encoding GFP were isolated or detected from the luminal contents of all inoculated loops.

Gross and histopathology. Gross evidence of peritonitis was never observed. However, all sheep developed various degrees of adhesions, with adhesion foci often occurring at sutures. All anastomosis sites were well-healed (externally and internally) and showed no evidence of leakage or inflammation. In addition, the intestinal segment containing the catheterized loops, interspaces, and terminal spaces (that is, the 2 ends of the 85-cm segment of ileum) was healthy in appearance. Catheterized loops and interspaces were slightly distended in some instances and contained soft casts of sloughed epithelial cells. Mucosal surfaces of all loops and interspaces appeared normal.

Histologic samples from catheterized loops, interspaces, and intact ileum were blind-scored for evidence of tissue change. In all instances, scores were low, with no differences among catheterized loops, interspaces, or intact ileum. Scores (0 to 3; mean ± SEM) were 0.07 ± 0.04 to 0.13 ± 0.06 for necrosis, 0.20 ± 0.03 to 0.33 ± 0.07 for inflammation, 0.20 ± 0.06 to 0.44 ± 0.19 for villus blunting, 0.02 ± 0.02 to 0.10 ± 0.10 for lympholysis, and 0.0 to 0.20 ± 0.13 for fibrosis.

Expression of cytokine and iNOS genes. We designed and validated several new primers for quantification of ovine genes involved in inflammation, including primers for the quantification of IFNγ, IL4, IL18, TNFα, TGFβ1, iNOS, and the housekeeper gene β2 microglobulin (Table 1). We were unable to consistently amplify a product by using the published primer sets for IL1β, and this primer set was discarded. We determined that GAPDH was the appropriate housekeeper gene to normalize our data (that is, GAPDH exhibited a cycle threshold in the range similar to the sheep samples, whereas the cycle threshold for the β2 microglobulin gene was dissimilar to sheep samples). Consistent with gross and microscopic evaluations, expression of genes for cytokines or iNOS showed no significant differences ($P > 0.05$) among the 3 tissue types, with considerable variability in expression among the 5 sheep (Table 2).

Tissue electrical resistance. Electrical resistance showed no significant differences among catheterized loops, interspaces, and intact ileum) alone or as a function of time (Figure 1). The intestinal epithelium exhibits both polarity and tightness. Polarity is generated by the asymmetric distribution of proteins to either the apical or the basolateral membranes and tightness is due to the permeability of tight junctions.[24] Therefore, measures of electrical resistance provide an indication of the integrity of epithelial tissues.

Microbiota. To evaluate the effect of antibiotics on the microbiota, we used culture-independent methods. Using quantitative PCR for eubacterial DNA, we determined that the total number of bacterial cells was reduced in catheterized loops ($P < 0.01$) and interspaces ($P < 0.05$) relative to the adjacent intact ileum (Figure 2). However, neither the catheterized loops nor interspaces were rendered sterile by the administration of the broad-spectrum and bacteriocidal antibiotics metronidazole, enrofloxacin, and ampicillin.

Table 2. Relative expression ratios (mean ± SEM; $n = 5$) of proinflammatory cytokine and iNOS genes in mucosal tissues obtained from catheterized loops, interspaces, and intact ileum after normalization with GAPDH by using the $2^{-\Delta\Delta CT}$ method

Gene	Catheterized loops	Interspaces	Intact ileum
IFNγ	2.4 ± 0.62	1.1 ± 0.28	3.0 ± 0.66
IL1α	11.2 ± 6.03	7.9 ± 5.82	1.1 ± 0.29
IL4	1.3 ± 0.42	2.1 ± 0.54	4.5 ± 2.41
IL6	16.3 ± 5.54	6.6 ± 1.72	9.9 ± 8.54
IL12 p40	1.5 ± 0.18	2.0 ± 0.36	1.2 ± 0.30
IL18	1.2 ± 0.28	2.7 ± 1.24	0.8 ± 0.12
TGFβ1	1.9 ± 0.33	2.0 ± 0.79	1.0 ± 0.18
TNFα	2.4 ± 0.66	1.2 ± 0.30	1.1 ± 0.41
iNOS	3.7 ± 3.07	12.3 ± 11.19	42.4 ± 14.28

Expression rates among tissue types by gene were not different ($P > 0.05$).

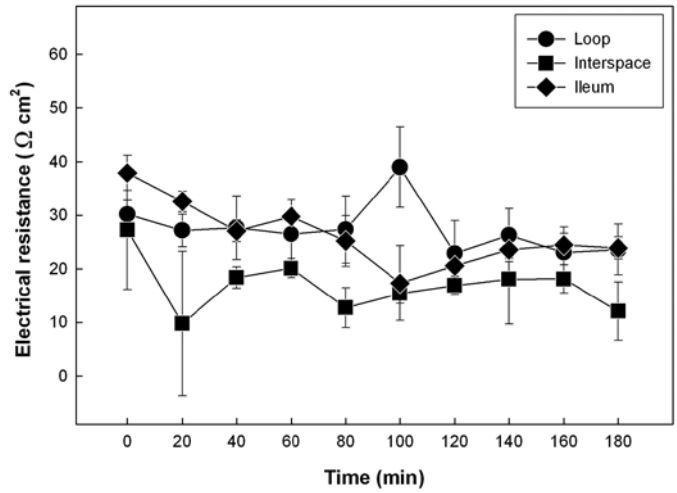

Figure 1. Tissue resistance (Ω cm^2) for catheterized loops, interspaces, and intact ileum as a function of interval time after harvest. Values are the means of 4 sheep (2 observations per replicate), and vertical bars associated with means represent the SEM. There was no interaction between treatment and time, and neither loop nor interspace differed ($P > 0.05$) from intact ileum averaged across time.

To further examine the effect of antibiotic administration on the microbiota, bacterial community structures were examined by using T-RFLP in conjunction with group significance and nonmetric multidimensional scaling analyses. The composition of mucosa-associated bacterial communities within loops and interspaces differed ($P = 0.066$ and $P = 0.051$) from that of the intact ileum, but not substantively (Figure 3 A). Consistent with these observations, a majority of the T-RF were shared among the 3 tissue types ($n = 116$), however 193 T-RF were detected in association with mucosa in the intact ileum compared with 163 and 160 T-RF in catheterized loops and interspaces, respectively (Figure 4). The relative abundance of the community constituents were highly affected ($P < 0.001$) by antibiotics (Figure 3 B).

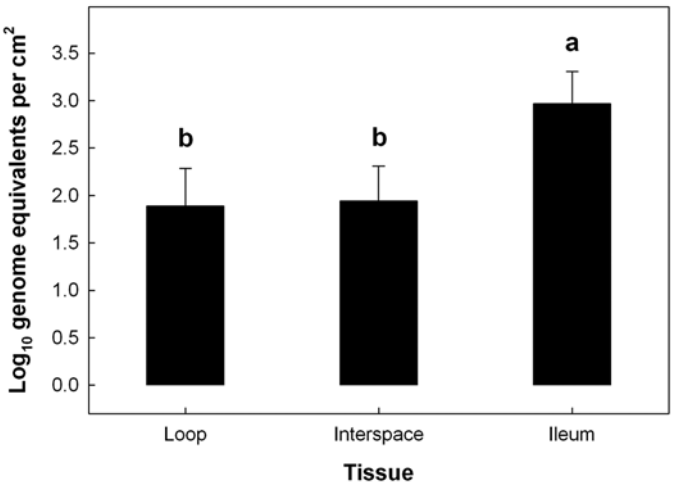

Figure 2. Populations of mucosa-associated eubacteria from catheterized loops, interspaces, and intact ileum determined with quantitative PCR. Samples were treated with EMA before DNA extraction (that is, to exclude amplification of cells not possessing intact cell membranes). Values are the means of 4 sheep (2 subsamples per replicate), and vertical bars associated with means represent the SEM. Different lowercase letters indicate values that differ significantly ($P < 0.05$).

Discussion

We surgically created intestinal loops from functional ileum (defined by the presence of continuous Peyer patches) of sheep.[18,28] Although the region proximal to the ileocecal fold is considered jejunum anatomically, the region of continuous Peyer patches can be present as much as 2 m proximal to the ileocecal fold, highlighting the importance of considering immunologic function when studying host responses. The salient advantage of the catheterization method[36] relative to other loop methods (for example, reference 16) is that sheep recover fully from the surgery and are able to clear all pharmaceuticals before treatments are introduced into the loops. Thus, this technique removes a potential confoundment for the interpretation of host responses (for example, measure of bacteria-induced inflammatory responses could be confounded by concurrent administration of analgesics and antibiotics). Sheep resumed normal activities approximately 5 to 7 d after surgery. Catheters remained firmly secured within the ileal loops and were patent for more than 40 d. Moreover, treatments (that is, bacteria) readily were deposited into the loops through the catheters, and administered bacteria remained localized within loops.

An important aspect of the study was to ascertain whether the insertion of catheters would adversely affect the health of sheep or induce abnormal changes in tissues. Neither surgery nor catheters imparted any long-term effects on the sheep, and sheep behavior and overall health were normal after the animals recovered from surgery. Furthermore, we found no discernable changes in tissue morphology or to epithelial integrity, and no significant differences in gene expression of cytokines (IFNγ, IL1α, IL4 , IL6, IL12p40, IL18, TGFβ1, TNFα) or iNOS among the 3 tissue types. The proteins encoded by these genes are involved in events associated with immune and intestinal function, including leukocyte recruitment, cell apoptosis, induction of naïve CD4+ T cells into mature differentiated T-helper cells, regulation of effector signals from cells involved in innate immunity, and intestinal inflammation.[4,6,23,27]

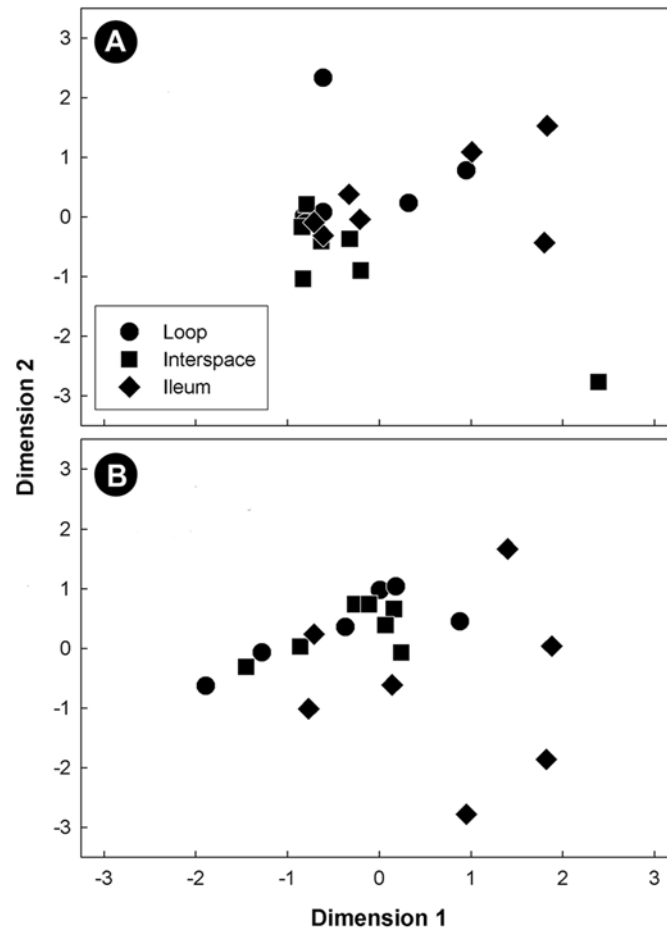

Figure 3. Nonmetric multidimensional scaling plots of mucosa-associated bacterial communities from catheterized loops, interspaces, and intact ileum of 4 sheep. Community structures were measured by using T-RFLP analysis on DNA treated with EMA. Nonmetric multidimensional scaling plots of T-RF (A) diversity and (B) relative abundance. Nonmetric multidimensional scaling is an ordination technique that arranges samples in multidimensional space on arbitrary axes according to their similarity or dissimilarity (that is, samples that are highly similar appear close together, whereas less similar samples are further apart).

In several aspects, the present findings were consistent with previous results in calves.[2] That study[2] used cDNA microarrays to examine whether "elimination of the microflora" after surgery altered gene expression in noncatheterized ileal loops of 1-mo-old Holstein calves. Although immunohistochemistry (that is, cytokeratin and a mucosal epithelial cell-specific antigen, RSK2-55) detected no significant differences between the loops and ileum 2 or 12 d after surgery, 2.3% of the expressed sequence tags were differentially expressed 2 d after surgery. Although the majority of expressed sequence tags returned to baseline levels by 12 d, some genes important for normal mucosal function remained differentially expressed. These observations emphasize the importance of allowing animals to fully recover from the surgical procedure, a key advantage of the catheterized intestinal loop model.

In light of previous studies using intestinal loop models, [2,16] we concluded that broad-spectrum antibiotics placed in the intestinal

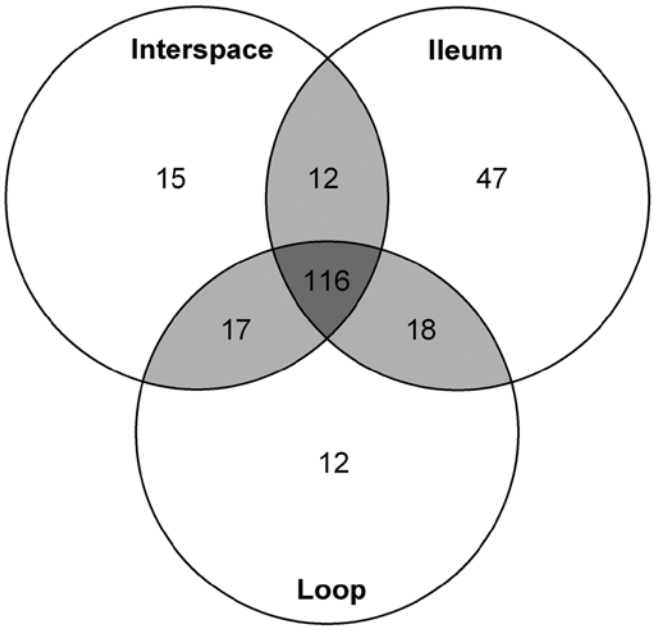

Figure 4. Three-way Venn diagram of T-RF from catheterized loops, interspaces, and intact ileum of 4 sheep. Data were combined across replicate animals and subsamples.

loops and interspaces during the surgical procedure rendered them sterile. The ability of antibiotics to kill bacteria within loops is important for subsequent use of the model to study host–microbiota interactions (that is, as an axenic or gnotobiotic model). To evaluate the effect of antibiotics on the microbiota, we used culture-independent methods. A salient criticism of PCR-based methods is the inability to determine whether amplified DNA originated from living or dead cells. Therefore, tissues were treated with EMA before DNA extraction. This method relies on the ability of EMA to penetrate the cell membrane of dead cells and covalently bind to DNA after irradiation, thereby preventing the annealing of primers to DNA. Both EMA and propidium monazide have successfully been used to quantify bacterial DNA from living cells.[15,21] Although comparative quantification of bacteria using quantitative PCR that targets universal genes may be subject to differential PCR bias among samples (for example, due to differences in bacterial community composition and unequal copy numbers of the 16S rRNA gene among bacteria), quantitative PCR analysis clearly indicated that loops and interspaces were not rendered sterile by broad-spectrum bacteriocidal antibiotics in the present study. In contrast, another study[16] used the same antibiotic regimen yet concluded that jejunal loops established in sheep were sterile. However, those authors assessed sterility by using light microscopy in conjunction with silver staining. Our contrasting results may be due to the differences in the location in which loops were established (that is, ileum versus jejunum) or, more likely, due to the increased sensitivity of molecular methods. Regarding the first possibility, the ileum of mammals has a well-developed microbiota relative to the proximal small intestine.[19] With respect to the second possibility, the vast majority of the bacteria within the intestine are recalcitrant, and it is generally accepted that nonmolecular-based methods, including light microscopy examinations, grossly underestimate microbial diversity within the intestine. Reports that broad-spectrum antibiotics sterilized the intestine of animals invariably have used culture-dependent methods.[22]

The administration of broad-spectrum antibiotics can cause reproducible short- and long-term effects on the intestinal microbiota.[30] A variety of factors, including the spectrum of activity of antibiotics, dosage, route of administration, duration of administration, and pharmacokinetics, all are thought to influence the degree of perturbation imparted by antibiotics. The microbiota of the intestinal tract of ruminants is relatively poorly understood. That 237 unique T-RF were detected in the sheep ileum in the present study provided clear evidence of a diverse microbiota. Furthermore, because only a single restriction enzyme was used, an individual T-RF can represent more than one taxon. Therefore, the current T-RF data were a conservative estimate of bacterial diversity. The small intestine is considered to have a less diverse community than the large intestine in mammals, but the cecum of beef cattle harbors an extensive microbiota comparable in community structure to that of the colon.[20] Presumably, bacterial exchange readily occurs between the cecum and ileum (through the ileocecal valve). Therefore, the presence of continuous Peyer patches within the ileum is consistent with the substantively higher densities of bacteria and more complex bacterial communities in the ileum relative to the jejunum of cattle[20] and other ruminants. Although antibiotic administration did not substantively affect the composition of the community in the present study, it selectively favored particular taxa, as indicated by the significant changes in relative abundance of T-RF. Because rearing germ-free ruminants is difficult, the ability to sterilize loops will be a valuable tool for studying host–microbiota interactions. In that regard, the evaluation of other antibiotics and administration strategies to render loops sterile has been initiated by our group. The administration of antibiotics into the loops through the catheters may facilitate the ability of antibiotics to sterile the loops. However, due to the recognized effects of antibiotics on the microbial ecology of the intestine (for example, bacterial overgrowth and antibiotic-associated diarrhea) coupled with secondary effects of antibiotics on the host (for example, as immunomodulators), the use of antibiotics for loop sterilization must be evaluated carefully.

In conclusion, we previously described a novel surgical method in which catheters were established in intestinal loops of sheep as a ruminant model.[36] The goal of this surgical method was to allow inoculation of loops once sheep had fully recovered from surgery, thus avoiding the presence of antibiotics and analgesics, which could confound the study of the host–microbiota–bacterial pathogen interaction. Catheterization of ileal loops did not affect the recovery of animals from surgery, and animals functioned normally throughout the postsurgery period (more than 40 d). Catheters remained patent, and GFP *E. coli* cells were deposited into the loops through the catheters and remained localized within the loops. The catheters did not cause any tissue injury and did not affect intestinal integrity. Contrary to previous reports,[1,2,16,26] administration of broad-spectrum antibiotics did not sterilize to loops or interspaces. Furthermore, antibiotics did not substantively affect the composition of the microbiota within loops or interspaces, but they did change the relative abundance of community constituents.

Acknowledgments

The research could not have been completed without the contributions of several persons at the AAFC Lethbridge Research Centre. We thank Tara Shelton for pre- and postoperative care of animals, surgical preparation, anesthesiology, conducting health assessments, inoculating loops with GFP *E. coli*, tissue harvest, quantification of GFP *E. coli*, tissue preparation for histology extraction, and conducting RT-qPCR; Jenny Gusse for preparing the GFP *E. coli* inoculum, designing and validating primers, conducting Ussing chamber analyses, extracting DNA, conducting qPCR of eubacterial cells, conducting the T-RFLP analysis, and assisting with the T-RFLP statistical analyses; Randy Wilde for pre- and postoperative care of animals and tissue harvest; Estela Costa for pre- and postoperative care of animals and tissue harvest; Matt Thomas for conducting the nonmetric multidimensional scaling analyses of the T-RFLP data; Lisa Kalischuk for providing advice on the analysis of the cytokine gene expression data; Brian Egland for sheep husbandry and Toby Entz for providing experimental design and statistical analysis advice. We also thank Bill Miller (US Department of Agriculture–ARS, Albany, CA) for providing the GFP construct, and Donna Vine (Department of Agricultural, Food and Nutritional Science, University of Alberta) and Veronica Swystun and Christina Hirota (Department of Physiology and Biophysics, University of Calgary, Calgary, Canada) for providing advice and training on Ussing chamber analysis. This work was supported in part by grants from the Advanced Foods and Materials Network, the Alberta Livestock Industry Development Fund, the Canada-Alberta Beef Industry Development Fund, and Agriculture and Agri-Food Canada.

References

1. **Aich P, Wilson HL, Kaushik RS, Potter AA, Babiuk LA, Griebel P.** 2007. Comparative analysis of innate immune responses following infection of newborn calves with bovine rotavirus and bovine coronavirus. J Gen Virol **88:**2749–2761.
2. **Aich P, Wilson L, Rawlyk NA, Jalal S, Kaushik RS, Begg AA, Potter AA, Babiuk LA, Abrahamsen MS, Griebel PJ.** 2005. Microarray analysis of gene expression following preparation of sterile intestinal 'loops' in calves. Can J Anim Sci **85:**13–22.
3. **Alluwaimi AM, Cullor JS.** 2002. Cytokines gene expression patterns of bovine milk during middle and late stages of lactation. J Vet Med B Infect Dis Vet Public Health **49:**105–110.
4. **Amsen D, Spilianakis CG, Flavell RA.** 2009. How are TH1 and TH2 effector cells made? Curr Opin Immunol **21:**153–160.
5. **Angioi K, Hatier R, Merle M, Duprez A.** 2002. Xenografted human whole embryonic and fetal entoblastic organs develop and become functional adult-like micro-organs. J Surg Res **102:**85–95.
6. **Arend WP.** 2002. The balance between IL1 and IL1Rα in disease. Cytokine Growth Factor Rev **13:**323–340.
7. **Brun P, Castagliuolo I, Di Leo V, Buda A, Pinzani M, Palù G, Martines D.** 2007. Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. Am J Physiol Gastrointest Liver Physiol **292:**G518–G525.
8. **Canadian Council on Animal Care.** 1993. Guide to the care and use of experimental animals, vol 1, 2nd ed. Ottawa (Canada): Canadian Council on Animal Care.
9. **Castillo M, Martín-Orúe SM, Roca M, Manzanilla EG, Badiola I, Perez JF, Gasa J.** 2006. The response of gastrointestinal microbiota to avilamycin, butyrate, and plant extracts in early-weaned pigs. J Anim Sci **84:**2725–2734.
10. **Cilia V, Lafay B, Christen R.** 1996. Sequence heterogeneities among 16S ribosomal RNA sequences, and their effect on phylogenetic analyses at the species level. Mol Biol Evol **13:**451–461.
11. **Cooley MB, Miller WG, Mandrell RW.** 2003. Colonization of *Arabidopsis thaliana* with *Salmonella enterica* and enterohemorrhagic *Escherichia coli* O157:H7 and competition by *Enterobacter asburiae.* Appl Environ Microbiol **69:**4915–4926.
12. **Costa E, Puhl NJ, Selinger LB, Inglis GD.** 2009. Characterization of mucosa-associated bacterial communities of the mouse intestine by terminal restriction fragment length polymorphism: utility of sampling strategies and methods to reduce single-stranded DNA artifacts. J Microbiol Methods **78:**175–180.
13. **Coussens PM, Verman N, Coussens MA, Elftman MD, McNulty AM.** 2004. Cytokine gene expression in peripheral blood mononuclear cells and tissues of cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*: evidence for an inherent proinflammatory gene expression pattern. Infect Immun **72:**1409–1422.
14. **Culman SW, Bukowski R, Gauch HG, Cadillo-Quiroz H, Buckley DH.** 2009. T-REX: software for the processing and analysis of T-RFLP data. BMC Bioinformatics **10:**171.
15. **Delgado-Viscogliosi P, Solignac L, Delattre JM.** 2009. Viability PCR, a culture-independent method for rapid and selective quantification of viable *Legionella pneumophila* cells in environmental water samples. Appl Environ Microbiol **75:**3502–3512.
16. **Gerdts V, Uwiera RR, Mutwiri GK, Wilson DJ, Bowersock T, Kidane A, Babiuk LA, Griebel PJ.** 2001. Multiple intestinal 'loops' provide an in vivo model to analyse multiple mucosal immune responses. J Immunol Methods **256:**19–33.
17. **Golan L, Livneh-Kol A, Gonen E, Yagel S, Rosenshine I, Shpigel NY.** 2009. *Mycobacterium avium paratuberculosis* invades human small-intestinal goblet cells and elicits inflammation. J Infect Dis **199:**350–354.
18. **Griebel PJ, Davis WC, Reynolds JD.** 1992. An analysis of the growth and differentiation of B cells isolated from follicles of the ileal Peyer's patch of sheep. Immunology **75:**601–607.
19. **Hao WL, Lee YK.** 2004. Microflora of the gastrointestinal tract: a review. Methods Mol Biol **268:**491–502.
20. **Inglis GD.** 2010. Unpublished data.
21. **Inglis GD, McAllister TA, Larney FJ, Topp E.** 2010. Prolonged survival of *Campylobacter* species in bovine manure compost. Appl Environ Microbiol **76:**1110–1119.
22. **Johnson SA, Nicolson SW, Jackson S.** 2004. The effect of different oral antibiotics on the gastrointestinal microflora of a wild rodent (*Aethomys namaquensis*). Comp Biochem Physiol Part A Mol Integr Physiol **138:**475–483.
23. **Keklikoglu N, Koray M, Kocaelli H, Akinci S.** 2008. iNOS expression in oral and gastrointestinal tract mucosa. Dig Dis Sci **53:**1437–1442.
24. **Li H, Sheppard DN, Hug MJ.** 2004. Transepithelial electrical measurements with the Ussing chamber. J Cyst Fibros **3:**123–126.
25. **Lodemann U, Martens H.** 2006. Effects of diet and osmotic pressure on Na+ transport and tissue conductance of sheep isolated rumen epithelium. Exp Physiol **91:**539–550.
26. **Meurens F, Berri M, Auray G, Melo S, Levast B, Virlogeux-Payant I, Chevaleyre C, Gerdts V, Salmon H.** 2009. Early immune response following *Salmonella enterica* subspecies *enterica* serovar *Typhimurium* infection in porcine jejunal gut loops. Vet Res **40:**05.
27. **Mitsuyama K, Sata M, Rose-John S.** 2006. Interleukin-6 trans-signaling in inflammatory bowel disease. Cytokine Growth Factor Rev **17:**451–461.
28. **Motyka B, Griebel PJ, Reynolds JD.** 1993. Agents that activate protein kinase C rescue sheep ileal Peyer's patch B cells from apoptosis. Eur J Immunol **23:**1314–1321.
29. **Neuvians TP, Schams D, Berisha B, Pfaffl MW.** 2004. Involvement of proinflammatory cytokines, mediators of inflammation, and basic fibroblast growth factor in prostaglandin F2a-induced luteolysis in bovine corpus luteum. Biol Reprod **70:**473–480.
30 **Preidis GA, Versalovic J.** 2009. Targeting the human microbiome with antibiotics, probiotics, and prebiotics: gastroenterology enters the metagenomics era. Gastroenterology **136:**2015–2031.
31 **Santos RL, Zhang S, Tsolis RM, Baumler AJ, Adams LG.** 2002. Morphologic and molecular characterization of *Salmonella typhimurium* infection in neonatal calves. Vet Pathol **39:**200–215.
32. **Savidge TC, Pan W-H, Newman P, O'Brien M, Anton PM, Pothoulakis C.** 2003. *Clostridium difficile* toxin B is an inflammatory enterotoxin in human intestine. Gastroenterology **125:**413–420.
33. **Strandberg Y, Gray C, Vuocolo T, Donaldson L, Broadway M, Tellam R.** 2005. Lipopolysaccharide and lipoteichoic acid induce

different innate immune responses in bovine mammary epithelial cells. Cytokine **31:**72–86.

34. **Thulin JD, Kuhlenschmidt MS, Gelberg HB.** 1991. Development, characterization, and utilization of an intestinal xenograft model for infectious disease research. Lab Invest **65:**719–731.
35. **Ungell AL, Andreasson A, Lundin K, Utter L.** 1992. Effects of enzymatic inhibition and increased paracellular shunting on transport of vasopressin analogues in the rat. J Pharm Sci **81:**640–645.
36. **Uwiera RRE, Kastelic JP, Inglis GD.** 2009. Catheterization of intestinal loops in ruminants. J Vis Exp **28**. .
37. **Waldvogel AS, Lepage M-F, Zakher A, Reichel MP, Eicher R, Heussler VT.** 2004. Expression of interleukin 4, interleukin 4 splice variants and interferon γ mRNA in calves experimentally infected with *Fasciola hepatica.* Vet Immunol Immunopathol **97:**53–63.
38. **Yin X, Wheatcroft R, Chambers JR, Liu B, Zhu J, Gyles CL.** 2009. Contributions of O island 48 to adherence of enterohemorrhagic *Escherichia coli* O157:H7 to epithelial cells *in vitro* and in ligated pig ileal loops. Appl Environ Microbiol **75:**5779–5786.
39. **Yuan JS, Reed A, Chen F, Stewart CN.** 2006. Statistical analysis of real-time PCR data. BMC Bioinformatics **7:**85.