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Chronic shedding of *Campylobacter* species in beef cattle

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ABSTRACT

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Aims: To determine the prevalence of chronic shedding of *Campylobacter* species by beef cattle, a longitudinal study of shedding patterns was conducted in a cohort of 60 beef steers over a 4-month period.

Methods and Results: Steers were maintained in a simulated feedlot setting but individually in pens to minimize transmission among animals. At each collection time, campylobacters in faeces were detected using conventional PCR. In addition, quantities of *Campylobacter jejuni* and *C. lanienae* in faeces were measured using real-time quantitative (RTQ) PCR. All of the steers tested shed *Campylobacter* species during the course of the study, and overall, 90% of the 299 samples tested were positive for *Campylobacter* DNA. The majority of the animals (86%) shed campylobacters at ≥4 sample times. The most prevalent taxon detected in bovine faeces was *C. lanienae* (56% of samples) followed by *C. jejuni* (13%), *C. hyointestinalis* (8%), and *C. fetus* (2%). No *C. coli* was detected, and 13% of the faecal samples contained two or more of the above species. Seven (12%) and 34 (57%) animals shed *C. jejuni* and *C. lanienae* at ≥3 sample times, respectively. For both *C. lanienae* and *C. jejuni*, a substantial number of cells were detected in faeces using RTQ-PCR; 27% of the samples positive for *C. jejuni* contained populations $>10^4$ cells g^{-1} (maximum of 5×10^5 cells g^{-1}), and 44% of samples positive for *C. lanienae* possessed populations $>10^6$ cells g^{-1} (maximum of 4×10^8 cells g^{-1}). A significant correlation was observed between shedding of *C. lanienae* and the severity of liver abscesses. In 27% of the samples, an amplicon was obtained for genus-specific but not for the species-specific primers. Sequencing of the partial 16S rRNA gene suggested the presence of at least two undescribed *Campylobacter* species but this has yet to be confirmed.

Conclusions: A high percentage of feedlot cattle shed large quantities of *Campylobacter* species in their faeces over a protracted period of time (*ca* 112 days).

Significance and Impact of the Study: This is the first study of longitudinal shedding patterns of campylobacters in beef cattle using PCR-detection methods. In addition, this is the first use of RTQ-PCR to directly quantify *C. jejuni* or *C. lanienae* in faeces. The results of the study show that a large number of cattle (>85%) chronically shed campylobacters in feedlots.

Keywords: bovine, *Campylobacter*, longitudinal, quantitative PCR, shedding.

INTRODUCTION

Campylobacter species are recognized as one the most frequent causes of acute diarrhoeal disease in humans in North America (Centers for Disease Control and Prevention-U.S. Department of Agriculture-Food and Drug Administration Collaborating Sites Foodborne Disease Active Survey Network [Foodnet]). Alberta, Canada, possesses a very large beef cattle population (approx. 6 million head), and approx. 2 million of these animals are in finished feedlots (Alberta Government Website, http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/sdd1492?opendocument). Despite the large numbers of cattle concentrated in feedlots, relatively limited research has addressed the

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prevalence of *Campylobacter* species associated with beef cattle. Furthermore, the prevalence of *Campylobacter* infections in humans in Alberta is higher than the national average (Health Canada Website, http://dsol-smed.hc-sc.gc.ca/dsol-smed/ndis/index_e.html). The results of molecular typing of *C. jejuni* isolates in Europe has demonstrated a similarity between strains associated with cattle and those isolated from humans (On *et al.* 1998; Nielsen *et al.* 2000; Dingle *et al.* 2001; Fitzgerald *et al.* 2001; Schouls *et al.* 2003), but a relationship between high cattle densities and the high prevalence of campylobacteriosis has not been definitively established. However, waterborne *C. jejuni* from a bovine source were implicated in the infection of a large number of people at Walkerton, Ontario, Canada in 2000 (Clark *et al.* 2003). Mounting evidence is implementing cattle as a significant source of *Campylobacter* subtypes that incite disease in humans (Stanley and Jones 2003).

Shedding rates of campylobacters is typically higher in calves than in adults (Stanley and Jones 2003). However, a PCR-based 'snapshot' survey of 380 steers in a finishing feedlot demonstrated that a very high number (83%) of these animals were shedding campylobacters (Inglis *et al.* 2003). The most prevalent taxa detected were *C. lanienae* (49%) and *C. jejuni* (38%); *C. lanienae* could not be cultured on commonly used media (Inglis and Kalischuk 2003). Although *C. jejuni* infects or is associated with the gastrointestinal (GI) tract of numerous animals, the duration of shedding in individuals has not been extensively studied in most species. Immunocompetent adult humans do not typically shed *C. jejuni* for protracted periods of time (Skirrow 1994). In contrast, prolonged bouts of shedding (approx. 43 days) have been observed in poultry (Achen *et al.* 1998). A seasonality of shedding on a herd basis has been documented in cattle (Stanley *et al.* 1998), but relatively little is known about the longitudinal shedding of *Campylobacter* species in individual bovine. However, in a study of a small number of dairy cows (≤29 animals) using conventional isolation methods, Hanninen *et al.* (1998) observed that a single dairy cow shed *C. jejuni* at four of eight sample times over a 16-month period; they designated this animal as a 'permanent colonizer'. The prevalence of shedding and whether individual bovine become chronic shedders of enteric pathogens has important consequences on assessing risk and on the design and implementation of management strategies. For example, research is currently being directed towards the identification of beef cattle that shed verotoxigenic *Escherichia coli* serotypes immediately prior to shipment so that these animals can be eliminated or handled appropriately in the processing facilities. Therefore, the objective of the current study was to measure the longitudinal shedding of a variety of *Campylobacter* species in a cohort of 60 Angus-cross beef steers over a 4-month period. The steers were maintained in a simulated feedlot setting (i.e. during the finishing period) with the exception that animals were restricted to individual pens to minimize transmission. PCR was used to detect and quantify *Campylobacter* species shed in faeces.

MATERIALS AND METHODS

Maintenance of steers and collection of faeces

As part of an on-going study to determine the effects of hemp seed on finishing performance, 60 Angus-cross steers (approx. 18 months old) were maintained in individual pens; the pens were constructed of 5×20 cm boards with a 0·20 cm gap between the boards, and two animals shared a common water trough. The same animal occupied a specific pen throughout the course of the study. Water was provided from the City of Lethbridge, and was chlorine-treated. Upon arrival at Lethbridge Research Centre (LRC), young steers were maintained on a transition diet in which the concentration of steam rolled barley grain was increased. The base finishing diet consisted of 9% barley silage, 86% barley grain, and 5% supplement containing vitamins and minerals; all percentages were calculated from dry weights. Steers were fed once per day. Twenty arbitrarily selected animals were assigned to each of three diet treatments, and the experiment was designed as a randomized complete block design with individual animals serving as blocks. The three diet treatments consisted of: a base barley finishing diet amended with 4·6% soya bean meal (treatment 1); the base barley diet amended with 9% hemp seeds (treatment 2); and the base barley diet amended with 14% hemp seeds (treatment 3). The amount of feed consumed each day was recorded. Steers were weighed at 28-day intervals (15 October, 13 November, 11 December, 8 January, and 5 February). To accomplish this, steers were allowed to leave their stalls and travel to an adjacent weigh station; steers were weighed in specific groups, and the order in which steers were weighed were recorded on each sample date. During weighing, faecal samples were obtained per rectum. New gloves were used for each sample. Faecal samples were placed in sterile urine collection cups, immediately placed on ice, and transferred to the laboratory within 2 h of collection. Arbitrarily selected aliquots of faecal material (200 ± 5 mg) were aseptically transferred into 2-ml tubes and frozen at −20°C until processed. After the last collection period, average daily dry matter intake, average daily gain, and feed efficiency were recorded for the finishing period. Cattle were transported to a federally inspected abattoir, and the following performance parameters were recorded: (i) carcass weight; (ii) dressing percentage; (iii) back fat thickness (mm); (iv) ribeye area (cm^2); (v) lean meat yield (%); (vi) marbling (three categories); and (vii) liver abscess severity (score of 0 to 2).

DNA extraction

Bovine faeces contains a number of PCR inhibitors. To remove inhibitors the QIAamp® DNA stool mini kit (Qiagen Inc., Mississauga, ON, Canada) was used to extract DNA from each faecal sample according to the manufacturer's protocol for isolation of DNA from stool for pathogen detection. Briefly, the procedure involves lysis of the bacterial cells within the faecal material in an ASL buffer (i.e. developed to remove inhibitory substances), adsorption of impurities to an InhibitEX reagent, and purification of the DNA on a spin column. To determine whether PCR inhibitors had been sufficiently removed, an internal control was used (Inglis and Kalischuk 2003). The internal control was constructed by deleting a fragment of the *C. jejuni* (ATCC 49943) 16S rRNA gene and was designed to amplify under the same PCR conditions as described for the *Campylobacter* genus primer set; however, amplification yielded a 475 bp instead of the 816 bp product. The internal control DNA was added to faeces prior to extraction at a level of 10 μl per 200 mg of faeces. The presence of either a genus-specific or internal control amplicon, either of which indicated adequate removal of PCR inhibitors, was assessed with a non-nested *Campylobacter* genus-specific primer set. DNA was stored at −20°C until processed.

Conventional PCR and electrophoresis

Nested and non-nested PCR were conducted according to Inglis and Kalischuk (2003). Initially samples were amplified with the *Campylobacter* genus primer set. If a *Campylobacter* genus and/or internal control amplicon was observed, extracted DNA was then amplified using nonmultiplexed *C. hyointestinalis* primer set, and multiplexed *C. coli*–*C. jejuni* and *C. fetus*–*C. lanienae* primer sets. The conditions for primary amplification were: one cycle at 95°C for 15 min; 35 cycles (i.e. genus primer set) or 25 cycles (i.e. species primer sets) for 30 s at 94°C, 90 s at the appropriate annealing temperature (Inglis and Kalischuk 2003), 60 s at 72°C; and extension for 10 min at 72°C. Mixtures consisted of a total volume of 20 μl containing 1X reaction buffer, 0·2 mmol l^{-1} dNTPs, 2 mmol l^{-1} $MgCl_2$, 0·5 μmol l^{-1} of each primer (Sigma-Genosys, Oakville, ON, Canada), 0·2 μg BSA (Promega, Madison, WI, USA), and 1 U HotStar *Taq* polymerase (Qiagen, Inc.). Each PCR reaction was performed with a total of 2 μl of extracted DNA. For nested and semi-nested amplification, the same reaction conditions were employed with the exception that 35 cycles were used, 1 μl of the reaction mixture from the primary amplification step was used as template, and BSA was not included in the reaction mixture. All PCR products (10 μl) were electrophoresed in a 2% TBE-agarose gel (Invitrogen Corp., Burlington, ON, Canada), visualized by staining with ethidium bromide and viewed under u.v. light. A 100 bp ladder (Promega) was used to size products. Genomic DNA obtained from pure cultures of each *Campylobacter* taxon served as positive controls.

DNA sequencing

The partial *Campylobacter* 16S rRNA gene was amplified and sequenced for 26 arbitrarily selected samples that were positive for *Campylobacter* genus but negative for *C. coli*, *C. fetus*, *C. jejuni*, *C. hyointestinalis* and *C. lanienae* (Fig. 1). In addition, five arbitrarily selected samples that also were positive for specific species were sequenced. For these samples, a PCR product was initially obtained with the C412F/C1228R primer set; the same amplification conditions as described above were used. The resulting PCR products (10 μl) were electrophoresed in a 2% TAE-agarose gel, and a 100 bp ladder (Promega) was used to size products. The PCR amplicons were then purified using the Qiagen QIAquick kit (Qiagen Inc.), and then sequenced in forward (C412F) and reverse (C1228R) using the ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA). Before sequencing, excess dye was removed with the Qiagen DyeEx Spin Kit (Qiagen Inc.). Contigs were constructed

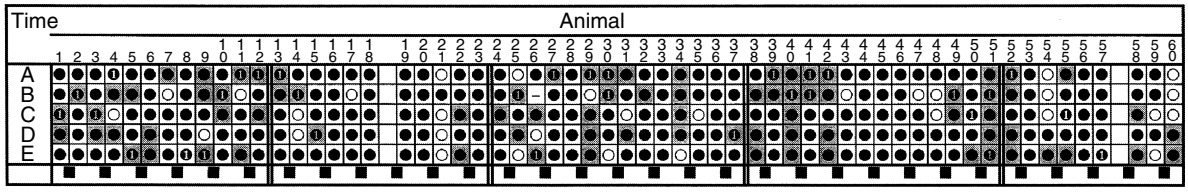

Fig. 1 Shedding patterns of *Campylobacter* species in faeces collected from 60 beef cattle maintained in a simulated feedlot on a barley finishing diet. Faecal samples were obtained at five times (28-day intervals) indicated by 'A' to 'E', and DNA was extracted and amplified with a genus-specific primer set (Inglis and Kalischuk 2003). Closed circles indicate positive detection of *Campylobacter* DNA, whereas, open circles indicate negative detection. Light grey boxes associated with positive samples indicate cases where samples that were positive for *Campylobacter*, but were negative for *C. coli*, *C. fetus*, *C. hyointestinalis*, *C. jejuni*, or *C. lanienae*. Closed circles containing a '1' indicate samples in which the *Campylobacter* genus amplicon was sequenced. Black boxes at the bottom of the graph indicate the location of water troughs, double vertical lines indicate the presence of a solid wall between pens, and the sample indicated by '–' is missing

using Staden (Medical Research Council, Laboratory of Molecular Biology, Cambridge, UK), and all sequences were compared directly with the NCBI GenBank nonredundant nucleotide database using BLASTN.

The nucleotide sequences were then aligned along with data retrieved from GenBank using the multi-alignment program ClustalW (Thompson *et al.* 1994), and the alignments were refined visually using GeneDoc (http://www.psc.edu/biome/genedoc/). The 16S rDNA sequences used were: *C. coli* (L04312); *C. concisus* (L04322); *C. curvus* (L04312); *C. fetus* (AJ306568); *C. fetus* subsp. *fetus* (M65012); *C. fetus* subsp. *venerealis* (M65011); *C. gracilis* (L04320); two strains of *C. hyointestinalis* (AF219235, M65010); three strains of *C. hyointestinalis* subsp. *hyointestinalis* (AF097689, AF097681, AF097691); three strains of *C. hyointestinalis* subsp. *lawsonii* (AF097683, AF097685, AF097688); seven strains of *C. lanienae* (AB076675, AB076677, AF043423, AF043424, AF043425, AF550664, AY165045), including one strain previously isolated from bovine in Lethbridge (AY288304); two strains of *C. jejuni* (CJE19244, L04315); *C. mucosalis* (L06978); *C. rectus* (L04317); and *C. sputorum* (L04319). Also included was the sequence for an uncultured bacterium (AF371867) from the GI-tract of a swine (Leser *et al.* 2002) which possessed high similarity to our sequences. The outgroup used was *Arcobacter cryaerophilus* (U25805). The sequence data were analysed using programs contained within PHYLIP (Felsenstein 1995). Phylogenetic estimates were obtained based on neighbour-joining distance, maximum parsimony, and maximum likelihood methods. Divergence (or distance) of each pair of sequences was calculated by DNADIST using Kimura's two-parameter model. The NEIGHBOUR program was used to carry out the neighbour-joining method for estimating phylogenies from the distance matrices. DNAPARS was executed to perform maximum parsimony, and DNAML was utilized to for maximum likelihood analysis. Support for the internal branches within the resulting trees was obtained by bootstrap analysis. A total of 1000 bootstrap replicates for the 16S rDNA data were generated by SEQBOOT, majority-rule consensus trees were constructed by the CONSENSE program, and the tree was visualized using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/rod.html). Evidence of chimeric sequences was assessed using Chimera Check relative to existing sequences within the Ribosomal Database Project II (Cole *et al.* 2003). In addition, the program Bellerophon that detects possible chimeric sequences in aligned datasets based on partial treeing analysis, was applied (Hugenholtz and Huber 2003).

Quantitative real-time PCR

Nested real-time quantitative (RTQ) PCR was applied to samples deemed to be positive for *C. jejuni* and *C. lanienae* as described previously (Inglis and Kalischuk 2004). For primary PCR, the primers MDmapA1Upper and MdmapA2Lower, and CLAN76F and CLANL521021R were used for *C. jejuni* and *C. lanienae*, respectively. The conditions for primary amplification were one cycle at 95°C for 15 min, followed by 22 (*C. lanienae*) or 25 (*C. jejuni*) cycles of 30 s at 94°C, 90 s at 58°C, and 60 s at 72°C, ending with an extension cycle of 10 min at 72°C. The same reaction mixture as indicated above was used. For nested RTQ-PCR, the primers QcjmapANF and QCjmapANR, and QclanN2F and QclanN2R were used for *C. jejuni* and *C. lanienae*, respectively. RTQ-PCR and melt curve analysis were conducted using an iCycler iQ^{TM} (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada). Reaction mixtures consisted of a total volume of 20 μl containing 1X QuantiTect^{TM} SYBR Green PCR Master Mix (Qiagen Inc.), 0·5 μmol l^{-1} of each primer, and 1 nmol l^{-1} of fluorescein (Bio-Rad). Each PCR reaction was performed with a total of 2 : 1 of template DNA. The conditions for amplification were one cycle at 95°C for 15 min, followed by 30–40 cycles of 15 s at 94°C, 30 s at 58°C, and 30 s at 72°C for data acquisition. Melt curve analysis was conducted over a range of 55–95°C, with increments set at 0·5°C for 10 s (80 cycles). In each run, standard samples were included to establish a standard curve. To prepare the quantification standard, *C. jejuni* and *C. lanienae* cells were scraped from the surface of the *Brucella* (Difco, Detroit, MI, USA) and Karmali (Oxoid, Nepean, ON, Canada) agars, respectively, after 48 h. DNA was extracted from the harvested cell mass using a DNeasy® kit (Qiagen Inc.) according to the manufacturer's protocol. DNA was measured fluorimetrically using a Hoefer DyNA Quant 200 apparatus (Amersham Biosciences Corp., Piscataway, NJ, USA); calf thymus DNA (Calbiochem, San Diego, CA, USA) was used as a standard. Genomic DNA standards for both bacteria were diluted in a 10-fold dilution series in 10 mmol l^{-1} Tris (pH 8·5). Standard DNA was thawed/frozen a maximum of two times. For both standard and unknown reactions, each sample was run twice and a mean value was used in all subsequent analyses.

Data were analysed using the iCycler iQT^{M} software (version 3.0; Bio-Rad). The user-defined 'PCR Base Line Subtracted' (i.e. a set of baseline cycles is applied globally to all samples) and 'Threshold Cycle Calculation' options were used to obtain the number of threshold cycles per well. Samples that produced no product or that produced an amplicon with an anomalous melting point were entered as missing. The linear equation for the standard curve (i.e. containing known quantities of DNA) was then used to interpolate the number of copies present in the unknown samples. The correlation coefficients for the standards averaged 0·97 (range from 0·94 to 0·99). To convert log

genome equivalents to log cells g^{-1}, the linear equations for this relationship (i.e. based on inoculated bovine faeces) were used (Inglis and Kalischuk 2004).

Statistical analyses

All analyses were conducted using Statistical Analyses System Software (SAS Institute Inc. 1999). For cattle performance characteristics, the experiment was analysed as a one-way analysis of variance arranged as a randomized complete block design with three levels of diet treatment and 20 levels of block using the mixed procedure of SAS. In no instance was a diet treatment effect ($F = 1{\cdot}5$; d.f. = 2, 37–56; $P = 0{\cdot}24$) observed for any of the parameters tested; diet treatments independent of *Campylobacter* shedding were therefore not considered further. To determine whether diet treatment affected quantities of total campylobacters, *C. jejuni* and *C. lanienae* shed in faeces at different times, the mixed procedure of SAS was used; the data were analysed as a factorial experiment with three levels of diet treatment and five levels of sampling time. As the same individual was used for all sample times, the repeated measurement statement was applied. The appropriate error structure was determined using Akaike's Information Criterion and Bayesian Information Criterion, and the Kenward-Roger degree of freedom feature was used to adjust the degrees of freedom of the error term. In conjunction with a significant *F*-test, the least square mean (LSMEAN) statement of SAS was used to produce the lsmeans and the DIFF option was applied to conduct the least significant difference test. Predicted values were plotted against residual values to determine whether residuals were randomly distributed, and the univariate procedure of SAS was applied to test for normality. Correlation analysis between amounts (mean across all five sample times) of *Campylobacter* genus (amplicon intensity), *C. jejuni* (cells g^{-1}), and *C. lanienae* (cells g^{-1}) in faeces with the various cattle performance characteristics were conducted using the correlation procedure of SAS; Spearman's rank correlation was applied for amplicon intensity, marbling, and liver abscesses (i.e. integer data). The influence of faecal shedding (*C. jejuni* and *C. lanienae*) between animals sharing and not sharing a water trough, at time x and time $x + 1$ (i.e. a time lag) also were assessed using the correlation procedure of SAS.

RESULTS

Campylobacter genus

In only one instance (animal 26 at collection time 2) of a total of 300 extractions did we not detect an amplicon for the internal control (i.e. 475 bp) or positive genus amplicon (i.e. 816 bp) using the *Campylobacter* genus primer set. A very high number of faecal samples were positive for campylobacters; overall, campylobacters were detected in 89·6% ($n = 268$) of the samples (Fig. 1). The frequency of shedding was consistent among the five sample times, and ranged from 84·7 to 93·3% (Fig. 2a). All of the animals shed *Campylobacter* at one or more of the sample times (Fig. 1). The majority of the animals (66·1%) shed campylobacters at all five sample times (i.e. over 4 months) and 20·3% were positive at four of five sample times (Fig. 3a).

A positive amplicon for specific species (i.e. six samples containing *C. lanienae* and one sample containing *C. hyointestinalis*) in the absence of a positive *Campylobacter* genus amplicon was observed in only 2·3% of the samples. In contrast, 27·4% ($n = 82$) of the samples positive for *Campylobacter* genus were negative for all of the species-specific primer sets tested (Fig. 1). Twenty-six of these amplicons were sequenced. BLASTN of the sequences obtained indicated that all possessed the greatest similarity with *Campylobacter* species (primarily *C. lanienae* and/or *C. hyointestinalis*) and/or with an unidentified bacterium from the GI tract of a swine (Leser *et al.* 2002). Phylogenetic analyses indicated that all were *Campylobacter* species, and all but one grouped most closely with *C. hyointestinalis* subsp. *lawsonii* and *C. lanienae*, but formed distinct clades from both of these taxa; at least two distinct clades were observed. Of the five arbitrarily selected samples that also were positive for *C. jejuni* or *C. hyointestinalis*, one sample grouped closely with *C. jejuni* (E8). However, the other four sequences (A4, C50, C55 and E57) did not. Interestingly, both E57 as well as E8 contained substantial numbers (>10^4 cells g^{-1}) of *C. jejuni* (Fig. 4). Although the 16S rDNA sequence data obtained was clearly from *Campylobacter* species, application of Chimera Check revealed that the sequences may be chimeric. In all cases, at least one of the sequence fragments possessed a closer ($S_{ab} = 0{\cdot}32$–$0{\cdot}97$) database relative (i.e. to *C. lanienae* and *C. hyointestinalis*) than the full-length sequence ($S_{ab} = 0{\cdot}79$–$0{\cdot}93$). Accession AF371867 also exhibited similar evidence of being chimeric. In contrast to the results obtained using Chimeric Check, analysis of aligned sequences using Bellerophon did not indicate that any of the 16S rDNA sequences were chimeric. We are now attempting to verify whether new taxa of *Campylobacter* species occur within in the GI tracts of cattle using a combination of molecular and classical methods.

Campylobacter jejuni

Overall, 13·4% of the faecal samples tested were positive for *C. jejuni* (Fig. 4) and the frequency of detection (range 8·2–16·7%) was relatively consistent across the five sample times (Fig. 2b). However, the amount of *C. jejuni* shed in faeces varied ($F = 2{\cdot}4$; d.f. = 8, 153; $P = 0{\cdot}016$) among the three

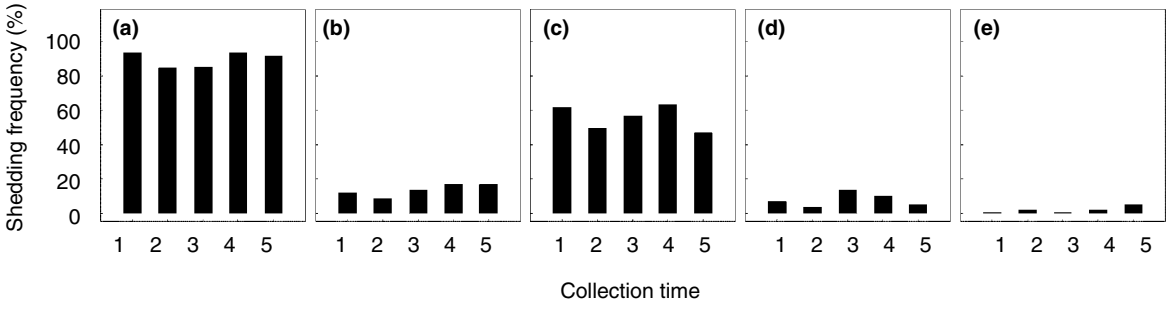

Fig. 2 Frequency (%) of shedding of *Campylobacter* genus and specific species in faeces collected from 60 beef cattle maintained in a simulated feedlot on a barley finishing diet by collection time. (a) *Campylobacter* species. (b) *C. jejuni*. (c) *C. lanienae*. (d) *C. hyointestinalis*. (e) *C. fetus*

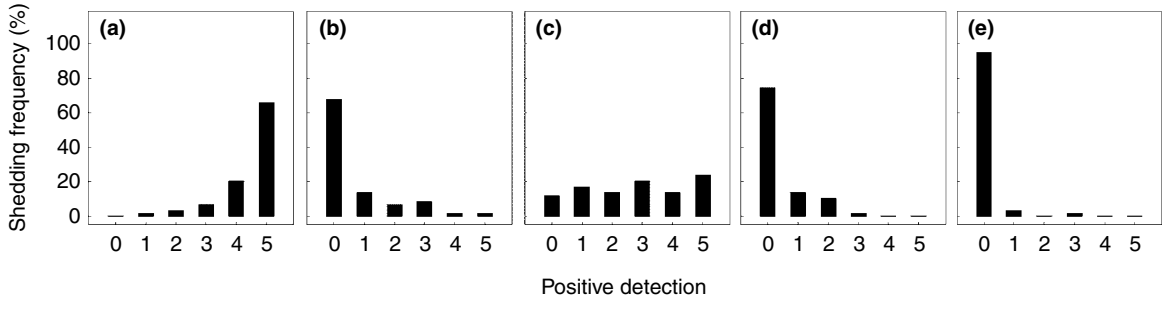

Fig. 3 Frequency (%) of beef cattle that shed *Campylobacter* genus and specific species in faeces as a function of the number of positive samples, where '0' is not detected and '5' is all samples were positive for *Campylobacter* DNA. (a) *Campylobacter* species. (b) *C. jejuni*. (c) *C. lanienae*. (d) *C. hyointestinalis*. (e) *C. fetus*

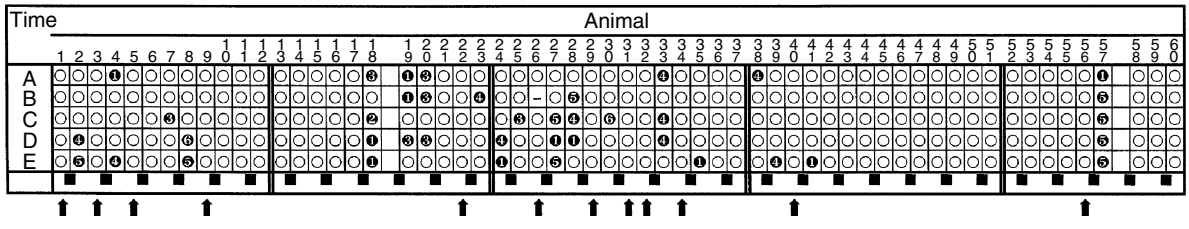

Fig. 4 Shedding patterns of *Campylobacter jejuni* in faeces collected from 60 beef cattle maintained in a simulated feedlot on a barley finishing diet. Faecal samples were obtained at five times (28-day-intervals) indicated by 'A' to 'E', and DNA was extracted and amplified with a nested *C. jejuni* primer set (Inglis and Kalischuk 2003). Positive detection of *C. jejuni* DNA is indicated by closed circles whereas, open circles indicate negative detection. All positive samples were subsequently subjected to real-time quantitative PCR targeting the single copy mapA gene (Inglis and Kalischuk 2004), and genome copies were quantified and converted to cells g^{-1} using the equation: $\log_{10}$ cells $g^{-1} = (\log_{10}$ genome copies + 1·49)/0·85. Numbers associated with dark circles indicate the density of *C. jejuni* in the faecal samples where: (1) <log 1 cells g^{-1} ; (2) >1 and <log 2 cells g^{-1} ; (3) >2 and <log 3 cells g^{-1} ; (4) >3 and <log 4 cells g^{-1} ; (5) >4 and <log 5 cells g^{-1} ; and (6) >5 and <log 6 cells g^{-1}. Black boxes at the bottom of the graph indicate the location of water troughs, double vertical lines indicate the presence of a solid wall between pens, and the sample indicated by '–' is missing. Arrows indicate animals that were negative for *C. jejuni* that were adjacent to individuals that shed large numbers of *C. jejuni* cells

diet treatments at the various sample times (Table 1), but no distinct trends were discerned. Of the 60 animals tested, 19 (31·7%) shed *C. jejuni* at some point during the course of the experiment. The faeces of one animal was positive for *C. jejuni* at all five sample times, and faeces from eight, four, five, and one animal were positive for the bacterium at one, two, three, and four sample times, respectively (Fig. 3b). Considerable numbers of *C. jejuni* cells were detected in faeces of numerous samples using RTQ-PCR of the single copy mapA gene (Fig. 4); 26·8% of the samples positive for *C. jejuni* possessed populations greater than 10^4 cells g^{-1} (maximum of 5×10^5 cells g^{-1}). Twelve animals adjacent to

Table 1 Mean *Campylobacter* densities in faeces ($\log_{10}$ cells g^{-1}) by collection time for feed treatments as determined by nested RTQ-PCR

Treatment/time	*C. jejuni*	*C. lanienae*
Treatment 1*		
1	0·35 (0·24)^{abcde}	3·78 (0·71)^{bcde}
2	0·0^{a}	1·95 (0·59)^{a}
3	0·32 (0·22)^{abcde}	2·39 (0·71)^{ab}
4	0·58 (0·33)^{bcde}	1·91 (0·63 ^{a}
5	0·78 (0·36)^{de}	2·22 (0·70)^{a}
Treatment 2†		
1	0·27 (0·18)^{abcd}	4·34 (0·74)^{de}
2	0·15 (0·14)^{abcd}	1·81 (0·62)^{a}
3	0·10 (0·10)^{abcd}	3·86 (0·71)^{bcde}
4	0·27 (0·18)^{abcd}	4·74 (0·70)^{e}
5	0·01 (0·01)^{ab}	4·29 (0·66)^{cde}
Treatment 3‡		
1	0·01 (0·01)^{ab}	2·30 (0·67)^{ab}
2	0·66 (0·37)^{cde}	2·45 (0·76)^{abc}
3	1·03 (0·42)^{e}	2·48 (0·72)^{abc}
4	0·39 (0·26)^{abcde}	2·57 (0·74)^{abcd}
5	0·44 (0·30)^{abcde}	1·47 (0·62)^{a}

Values in parentheses following mean values represent standard errors of the mean.

Mean values within columns not followed by the same letter are significantly different.

*Base barley diet alone (control).

†Base barley diet amended with 9% (w/w) hemp seeds.

‡Base barley diet amended with 14% (w/w) hemp seeds.

cattle that shed *C. jejuni* at a density $\geq 10^3$ cells g^{-1} were negative for the bacterium (Fig. 4; arrows). No correlation ($r = 0{\cdot}28$, $P = 0{\cdot}14$) was observed in numbers of *C. jejuni* shed in faeces between adjacent animals at time x or time $x + 1$. In addition, there were no correlations ($r \leq -0{\cdot}21$, $P \geq 0{\cdot}12$) between amounts of *C. jejuni* shed in faeces (averaged over time) and any of the cattle parameters tested.

Campylobacter lanienae

A high percentage of the faecal samples were positive for *C. lanienae* (55·5%) (Fig. 5). The frequency of detection ranged from 46·7 to 63·3% across the five sample times (Fig. 2c), and there was a significant interaction ($F = 3{\cdot}5$; d.f. = 8, 226; $P < 0{\cdot}001$) between diet treatment and sample time (Table 1). As with *C. jejuni*, there were no conspicuous trends in amounts of *C. lanienae* in faeces as a function of time. Fifty-three (88·3%) of the animals shed *C. lanienae* at least one sample time, and the faeces from 14 animals (23·3%) were positive for the bacterium at all five sample times (Fig. 3c). The faeces from 10, eight, 12 and eight animals were positive for *C. lanienae* DNA at one, two, three and four sample times, respectively (Fig. 3c). Based on RTQ-PCR of the 16S rRNA gene, large populations of *C. lanienae* were observed in many of the samples (Fig. 5). Of the samples positive for *C. lanienae* ($n = 166$), 44·0% possessed populations greater than 10^6 cells g^{-1} (maximum of 4×10^8 cells g^{-1}), and an additional 23·5% of the samples contained densities of *C. lanienae* of between 10^4 and 10^6 cells g^{-1}. In seven cases, no *C. lanienae* was detected in the faeces of animals that were housed in a stall adjacent to cattle that shed the bacterium (Fig. 5; arrows). Similarly to *C. jejuni*, there were no correlations ($r = 0{\cdot}34$, $P = 0{\cdot}09$) in numbers of *C. lanienae* shed in faeces between adjacent animals at time x or time $x + 1$. With the exception of liver abscesses in which a significant positive correlation was observed ($r = 0{\cdot}39$, $P = 0{\cdot}003$), there were no significant correlations ($r \leq -0{\cdot}19$, $P \geq 0{\cdot}14$) between amounts of *C. lanienae* shed in faeces (averaged over time) and all of the other cattle parameters tested.

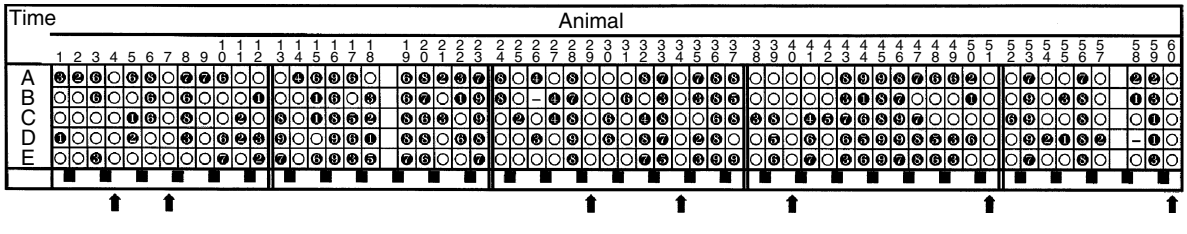

Fig. 5 Shedding patterns of *Campylobacter lanienae* in faeces collected from 60 beef cattle maintained in a simulated feedlot on a barley finishing diet. Faecal samples were obtained at five times (28-day intervals) indicated by 'A' to 'E', and DNA was extracted and amplified with a nested *C. lanienae* primer set (Inglis and Kalischuk 2003). Positive detection of *C. lanienae* DNA is indicated by closed circles whereas, open circles indicate negative detection. All positive samples were subsequently subjected to real-time quantitative PCR targeting the three copy 16S rRNA gene (Inglis and Kalischuk 2004), and genome copies were quantified and converted to cells g^{-1} using the equation: $\log_{10}$ cells $g^{-1} = (\log_{10}$ genome copies + 2·23)/0·97. Numbers associated with dark circles indicate the density of *C. lanienae* in the faecal samples where: (1) <log 1 cells g^{-1} ; (2) >1 and <log 2 cells g^{-1} ; (3) >2 and <log 3 cells g^{-1} ; (4) >3 and <log 4 cells g^{-1} ; (5) >4 and <log 5 cells g^{-1} ; (6) >5 and <log 6 cells g^{-1} ; (7) >6 and <log 7 cells g^{-1} ; (8) >7 and <log 8 cells g^{-1}; and (9) >8 and <log 9 cells g^{-1}. Black boxes at the bottom of the graph indicate the location of water troughs, double vertical lines indicate the presence of a solid wall between pens, and the sample indicated by '–' is missing. Arrows indicate animals that were negative for *C. lanienae* that were adjacent to individuals that shed large numbers of *C. lanienae* cells

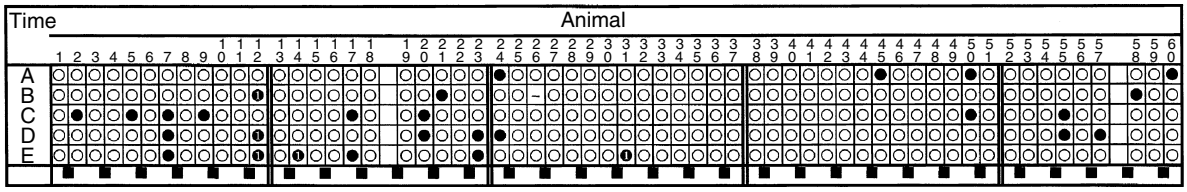

Fig. 6 Shedding patterns of *Campylobacter hyointestinalis* and *C. fetus* in faeces collected from 60 beef cattle maintained in a simulated feedlot on a barley finishing diet. Faecal samples were obtained at five times (28-day intervals) indicated by 'A' to 'E', and DNA was extracted and amplified with a semi-nested *C. hyointestinalis*-specific and nested *C. fetus*-specific primer sets (Inglis and Kalischuk 2003). Black circles indicate positive detection of *C. hyointestinalis* DNA, and black circles containing a '1' indicate positive detection of *C. fetus* DNA. Open circles indicate negative detection. Black boxes at the bottom of the graph indicate the location of water troughs, double vertical lines indicate the presence of a solid wall between pens, and the sample indicated by '–' is missing

Campylobacter hyointestinalis, *C. fetus* and *C. coli*

Relatively low numbers of samples were positive for *C. hyointestinalis* (7·7%) and *C. fetus* (1·7%) (Fig. 6), but the frequency of detection were similar among the five collection times (Fig. 2d,e). Of the 15 animals positive for *C. hyointestinalis*, one animal was positive at three of five sample times, and five and eight animals were positive at two and one sample time, respectively (Fig. 3D). With one exception, animals adjacent to those that shed *C. hyointestinalis* were free of the bacterium (Fig. 6). In total, only three animals shed *C. fetus*, and only one of these animals shed it greater than one sample time ($n = 3$) (Fig. 3e). As with *C. hyointestinalis*, adjacent animals were free of *C. fetus*. In no instance did we detect *C. coli*.

Multiple species

Thirty-nine samples (13·0%) contained greater than one species of *Campylobacter*, and 94·9% contained two taxa. The majority of these were positive for *C. jejuni* and *C. lanienae* (61·5%) and the remainder were positive *C. hyointestinalis* and *C. lanienae* (28·2%), *C. hyointestinalis* and *C. jejuni* (5·1%), and *C. fetus* and *C. lanienae* (5·1%). Only two samples were positive for three taxa (i.e. *C. hyointestinalis*, *C. jejuni* and *C. lanienae*).

DISCUSSION

A major thrust of many food safety programmes is risk assessment (i.e. the systematic process of understanding factors that influence the risk of adverse events such as the occurrence of food-borne illness). Considerable effort is now focussing on longitudinal studies of verotoxigenic *E. coli* serotypes associated with beef cattle within the risk assessment paradigm. In contrast to *E. coli* O157:H7, very limited research has addressed the potential risk associated with *Campylobacter* species from cattle, and even less effort has been directed towards a risk assessment of beef cattle in feedlots. As the first step in assessing the potential risk of *Campylobacter* species associated with beef cattle, we monitored shedding patterns in a cohort of 60 animals maintained in a simulated feedlot setting using PCR-based detection and quantification.

The majority of research conducted on *Campylobacter* species associated with livestock in relation to human disease has focussed on *C. jejuni*. Molecular typing of *C. jejuni* isolates in Europe have demonstrated a similarity between strains from cattle with those isolated from humans (On *et al.* 1998; Nielsen *et al.* 2000; Dingle *et al.* 2001; Fitzgerald *et al.* 2001; Schouls *et al.* 2003), and a recent outbreak of campylobacteriosis in Canada was tied to a bovine source (Clark *et al.* 2003). A number of 'snapshot' studies using microbiological methods (direct plating and/or enrichment) have demonstrated that cattle shed campylobacters, but carriage rates reported in these studies vary substantially. Variables such as herd size and type, season, age of the animals, sample site, sample frequency and isolation method, geography, diet and husbandry practices have all been implicated as significant parameters affecting carriage rates (Stanley and Jones 2003). In the current study, using direct PCR detection, we observed that faeces from all 60 of the beef cattle examined were positive for campylobacters, and overall, 90% of the samples obtained were positive. This is a substantially higher frequency than previously reported for faeces from adult cattle, and likely reflects the sensitivity of the molecular-based detection method used. However, such a high percentage of cattle that are positive for campylobacters is not without precedence. Using enrichment and direct plating of swabs from the small intestines of cattle, Stanley *et al.* (1998) observed that 89% of samples were positive for thermophilic campylobacters. In North America, beef cattle are typically maintained in high-density finishing feedlots for *ca* 6 months. Very limited information is available on the incidence of campylobacters in cattle in feedlots. However, Beach *et al.* (2002) observed that 64–68% of the faecal samples from feedlot cattle obtained pre- and post-transit to the abattoir were positive

for campylobacters by enrichment (i.e. using genus-specific enzyme-linked immunosorbent assay). In the current study, we observed that 19 of the 60 animals maintained in a simulated feedlot shed *C. jejuni* at one or more sample times over a 4-month period. Furthermore, we detected large numbers of *C. jejuni* (approx. 10^5 cells g^{-1}) in faeces of some animals using RTQ-PCR. As a 450 kg feedlot steer produces approx. 27 kg of manure per day (Queensland Government; http://www.dpi.qld.gov.au/environment/5166.html), numbers of *C. jejuni* released into the environment from a positive animal could exceed 10^9 cells per animal per day. The threshold of inoculum in humans is approx. 10–100 *C. jejuni* cells (Robinson 1981), and even a single animal shedding *Campylobacter* cells represents a potential risk.

Shedding of campylobacters by naturally colonized cattle is often intermittent (Stanley *et al.* 1998), but very limited information is available on whether individual cattle can become chronic shedders. Hanninen *et al.* (1998) observed that one of 29 dairy cows in herd shed *C. jejuni* at four of eight sample times over a 16-month period, and they designated this animal as a 'permanent colonizer'. In the current study, 12% (seven of 60) of the cattle examined shed *C. jejuni* at ≥3 sample times, and these animals were deemed to be chronic shedders. One animal in particular, was observed to shed very large numbers of *C. jejuni* cells at all five sample times. One proposed strategy is to cull 'high shedders' from the herd (Stanley and Jones 2003). Transmission of campylobacters among cattle via contaminated water has been demonstrated (Hanninen *et al.* 1998), and removing high shedders may limit transmission of *C. jejuni* within the herd or may prevent contamination of equipment and carcasses within the abattoir. In our study, animals were purposely maintained in individual pens and evidence suggested that minimal transmission occurred among adjacent animals. However, the density of cattle maintained in finishing feedlots are very high, and even one animal shedding *C. jejuni* may have a profound impact on the occurrence of this bacterium within the population. Few studies have addressed this possibility. However, the large number of animals that were observed to shed *C. jejuni* at one or more sample time in the finishing process would suggest that culling or isolating individuals shedding *C. jejuni* will not be economically feasible. Other management strategies may prove useful and these warrant investigation. The utility of management strategies will most likely rely on the ability to rapidly and accurately detect animals that are shedding high numbers of *Campylobacter* cells. The PCR-based technologies employed in the current study are accurate, sensitive, and rapid (Inglis and Kalischuk 2003, 2004) and they will facilitate the identification and implementation of efficacious management strategies.

The majority of cases of human enteritis are never connected to an aetiological agent (Mead *et al.* 1999), and the pathogenicity of fastidious species of *Campylobacter* such as *C. lanienae* is currently unknown. We observed that a very high percentage (88%) of cattle shed *C. lanienae*, and 57% of the 60 animals tested shed this bacterium at ≥3 sample times. Some of these animals were found to be shedding exceptionally large numbers of cells (approx. 10^8 cells g^{-1}), and numbers of *C. lanienae* released into the environment in cattle faeces could exceed 10^{12} cells $animal^{-1}$ day^{-1}. The densities of *Campylobacter* cells that we detected in cattle faeces using RTQ-PCR, are substantially higher than those reported using microbiological-based quantitation (i.e. typically in the 10^2–10^3 CFU g^{-1} range) (Nielsen 2002). All *Campylobacter* species are relatively fastidious, and our results clearly illustrate the utility of using RTQ-PCR. This is especially true for *C. lanienae*. This bacterium was originally isolated from the faeces of healthy abattoir workers in Switzerland (Logan *et al.* 2000); however, it has only recently been connected to cattle (Inglis *et al.* 2003; Inglis and Kalischuk 2003, 2004). Bovine strains of *C. lanienae* in Canada typically cannot be isolated on *Campylobacter* Charcoal Differential Agar (Oxoid) (Inglis and Kalischuk 2003), the most common medium used in hospitals to isolate campylobacters from human stool samples. Given that *C. lanienae* would not typically be detected in diagnostic facilities along with its prevalence in cattle faeces raises questions regarding its potential impact on human health.

Relative to *C. lanienae* and *C. jejuni*, a smaller number of cattle shed *C. hyointestinalis* (25%) and *C. fetus* (3%). *Campylobacter hyointestinalis* is only occasionally isolated from human patients with diarrhoea, and *C. fetus* subsp. *fetus* infrequently causes systemic infections in immunocompromised humans (Skirrow 1994). Based on sequencing of the 16S rRNA gene, Lawson *et al.* (1998) observed the presence of an uncultivated species, 'Candidatus Campylobacter hominis', in the GI tracts of healthy humans. We observed a large number of cattle faecal samples that were positive for *Campylobacter* genus by PCR, but were negative for all of the species-specific primer sets that we tested. Sequencing of the partial 16S rRNA gene suggested the presence of two potentially new taxa of *Campylobacter* in bovine faeces. However, the possibility that these sequences are chimeric for *C. lanienae* and *C. hyointestinalis* could not be ruled out, and the presence of novel species of *Campylobacter* in cattle faeces remains to be determined.

Whether *Campylobacter* species associated with adult cattle are pathogenic to cattle is not well understood. Beef cattle are typically administered subtherapeutic doses of antibiotics in North America to enhance performance, but the impact of these antibiotics on *Campylobacter* species in the GI tract of cattle are largely unknown. The inoculation

of calves with several *Campylobacter* species, including *C. coli*, *C. jejuni*, *C. fetus* subsp. *fetus*, *C. sputorum* subsp. *fecalis*, and *C. hyointestinalis* has been shown to incite mild to moderately severe enteritis in some animals (Al-Mahat and Taylor 1980, 1981, 1983; Warner and Bryner 1984; Terzolo *et al.* 1987; Diker *et al.* 1990), but adult cattle typically remain asymptomatic. Diarrhoea in humans is often malabsoptive in nature, but adult cattle possess an ability to absorb enormous quantities of water in their colon (Hecker and Grovum 1975), which may explain why they remain asymptomatic. Furthermore, it is possible that the site of infection of bovine by campylobacters is the colon. We also observed a significant correlation between *C. lanienae*, and the severity of liver abscesses. *Campylobacter jejuni* was isolated from 12% of the livers sampled from slaughtered cattle (Garcia *et al.* 1985), and the liver lesions that we observed were similar in appearance to those formed in mice that were inoculated intraperitoneally with *C. jejuni*; these mice also did not exhibit clinical symptoms of illness (Vuckovic *et al.* 1998). The impact of *Campylobacter* species on cattle as pathogenic (i.e. both intestinally and extra-intestinally) and/or commensalistic micro-organisms warrants study.

In conclusion, we found that 100% of the steers monitored over 4 months in a simulated feedlot setting shed campylobacters. Furthermore, a high percentage of these animals chronically shed *Campylobacter* species. In particular, large numbers of *C. lanienae* and *C. jejuni* cells were excreted in faeces from some animals. Evidence also suggested that *C. lanienae* may be pathogenic to cattle and that novel species of *Campylobacter* may occur within their GI tracts. Given the connection between bovine strains of *C. jejuni* and campylobacteriosis in humans (e.g. Clark *et al.* 2003), the large numbers of campylobacters being shed in faeces of feedlot cattle is of considerable concern. Furthermore, the high number of animals that are chronic shedders of campylobacters indicates that strategies proposed for other enteric pathogens associated with beef cattle (e.g. culling positive animals immediately prior to shipment) may not be an effective strategy to adequately reduce the risk associated with campylobacters shed in cattle faeces. The application of proactive measures that reduce the colonization of bovine GI tracts will likely be required.

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