Comparative Effect of Direct-Fed Microbials on Fecal Shedding of *Escherichia coli* O157:H7 and *Salmonella* in Naturally Infected Feedlot Cattle

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ABSTRACT

The effect of direct-fed microbials (DFM) on fecal shedding of *Escherichia coli* O157:H7 and *Salmonella* in naturally infected feedlot cattle was evaluated in a clinical trial involving 138 feedlot steers. Following standard laboratory methods, fecal samples collected from steers were evaluated for change in the detectable levels of *E. coli* O157:H7 and *Salmonella* shed in feces after DFM treatment. Sampling of steers was carried out every 3 weeks for 84 days. A significant reduction (32%) in fecal shedding of *E. coli* O157:H7 (*P* < 0.001), but not *Salmonella* (*P* = 0.24), was observed among the treatment steers compared with the control group during finishing. The probability of recovery of *E. coli* O157:H7 from the feces of treated and control steers was 34.0 and 66.0%, respectively. Steers placed on DFM supplement were almost three times less likely to shed *E. coli* O157:H7 (odds ratio, 0.36; 95% confidence interval, 0.25 to 0.53; *P* < 0.001) in their feces as opposed to their control counterparts. The probability of recovery of *Salmonella* from the feces of the control (14.0%) and the treated (11.3%) steers was similar. However, the DFM significantly reduced probability of new infections with *Salmonella* among DFM-treated cattle compared with controls (nontreated ones). It does seem that DFM as applied in our study are capable of significantly reducing fecal shedding of *E. coli* O157:H7 in naturally infected cattle but not *Salmonella*. The factors responsible for the observed difference in the effects of DFM on *E. coli* O157:H7 and *Salmonella* warrants further investigation.

Foodborne illnesses are a substantial health burden in the United States (24). Shiga toxin–producing *Escherichia coli* O157:H7 (STEC O157) and *Salmonella* play a significant role in foodborne infections. In humans, *E. coli* O157: H7 causes acute hemorrhagic colitis with a small percentage resulting in hemolytic uremic syndrome (23), whereas *Salmonella* causes salmonellosis. According to the 2006 FoodNet report (5), the incidence of infections caused by STEC O157 and *Salmonella* did not decrease significantly when compared with baseline data from the period of 1996 to 1999. This indicates that further measures are needed to prevent foodborne illness in order to achieve national health objectives. In 2006, a total of 17,252 laboratory-confirmed cases of infections in FoodNet surveillance areas were identified, of which 6,655 (38.57%) were determined to be associated with *Salmonella* (7). In addition, an estimated 95% of salmonellosis cases involved pathogen transmission mainly by foods of animal origin such as beef (4).

Cattle have been identified as a significant reservoir for *E. coli* O157:H7 (15), and *E. coli* O157:H7–related illness in humans is frequently associated with consumption of undercooked ground beef (3). Contamination of beef carcasses with *E. coli* O157:H7 has been linked to the presence of this organism on hides and in feces of cattle at the time of harvest (1, 9, 10). Also, an association has been reported between *Salmonella* shedding in cattle feces and the prevalence of *Salmonella* on cattle hides and other external surfaces (17). Given that cattle and their products are associated with the majority of cases of *E. coli* O157:H7 and *Salmonella* infections in humans, they represent an attractive target for preharvest intervention as a means of reducing risk to humans (23).

Effective intervention technologies implemented at preharvest to decrease the proportion of cattle carrying *E. coli* O157:H7 and *Salmonella* into slaughter plants may reduce the risk of human exposure to these organisms (29). Numerous preharvest intervention strategies have shown promising results in reducing the prevalence of *E. coli* O157:H7 in cattle including the use of direct-fed microbials (DFM) (2, 3, 8, 22, 28, 30). In addition to reducing the shedding of *E. coli* O157:H7, DFM (*Lactobacillus acidophilus* NP 51) has been reported to exert a dose-response effect on *Salmonella* shedding in cattle (26). The objective of this study was to evaluate the efficacy of feeding DFM *L. acidophilus* (LA 51) and *Propionibacterium freudenreichii* (PF 24) on fecal shedding of *E. coli* O157:H7 and *Salmonella* in naturally infected feedlot cattle.

MATERIALS AND METHODS

Study design. This was a clinical trial conducted for a period of 84 days from March 2007 through June 2007. One hundred
thirty-eight steers, initially weighing between 220 and 560 kg, were used in the study. The steers were divided into three blocks with the initial mean body weight as the blocking factor. Blocking by weight was aimed at targeting steers that will eventually attain the required weight for slaughter. The three blocks each comprised eight pens (six steers per pen), with an average weight of 446 ± 48.2, 475 ± 44.0, and 494 ± 31.2 kg per steer for blocks 1, 2, and 3, respectively. The allocation of treatments to pens was done randomly. Within each block, two treatments (treatment 1 comprised steers fed DFM, and treatment 2 comprised steers that did not receive DFM) were used throughout the finishing period) were assigned randomly to eight blocks, making a total of 12 pens per treatment. In general, one-half of the pens in each block were placed on DFM-supplemented feed, whereas the other half was maintained on DFM-free feed only. All steers were housed at the North Dakota State University (NDSU) feedlot facility located in Fargo, North Dakota.

Housing and feeding of cattle. One hundred forty-four 1-year-old steers were received at the NDSU feedlot facility on 26 October 2006. The steers were housed in 24 pens (six steers per pen). Prior to the beginning of the study, 6 of 144 steers died due to various causes, leaving 138 steers in the study. The steers in both groups were initially (October 2006 through February 2007) fed the same the growers’ diet consisting of barley (41.82%), desugared molasses (5.0%), grass hay (15.0%), and mineral supplement (2.9%) with an approximate analysis composition of 7.1% ash, 13.9% crude protein, 21.0% neutral detergent fiber, 8.7% acid detergent fiber, and 40.6% starch on dry matter basis. Both diets were formulated to provide 27.5 mg of calcium, 44.0, and 494 mg potassium per kg of diet dry matter. The finishing diet, in addition, had 11.0 mg of tylosin (Elanco Animal Health, Indianapolis, Ind.) per kg of diet dry matter.

In treatment pens, steers were fed DFM-supplemented finishing diet containing 1 × 10^6 CFU of L. acidophilus (LA 51) and 1 × 10^6 CFU of P. freudenreichii (PF 24) (Rosell Probiotics, Inc., Montreal, Quebec, Canada) per g of feed daily through the finishing period. The DFM were stored under refrigeration and constituted daily just before mixing with the feed. Steers in control pens only received the finishing diet through the finishing period. The two groups were monitored for fecal shedding of E. coli O157:H7 and Salmonella.

Sampling procedure. Sampling of cattle was conducted in accordance with the guidelines established by the Institute for Animal Care and Use Committee (IACUC) following a previously described protocol (12). Briefly, each steer was restrained in a hydraulic chute, and about 20 g of feces was collected from the rectum. A new set of sterile polyethylene sleeve gloves were used between collection from subsequent steers. The feces were put into sterile plastic cups that were placed on ice before being transported to the laboratory at NDSU for processing. In addition, a sterile dry (cotton) swab was used to swab the dorsal mucosa of the rectoanal junction (RAJ). Each RAJ mucosal swab was placed into a 15-ml culture tube containing 3 ml of Trypticase soy broth (Difco, Becton Dickinson, Sparks, Md.) supplemented with cefixime and potassium tellurite (Dynal Biotech ASA, Oslo, Norway). Both the fecal samples and swabs were placed in ice-cold packs coolers before transport to the laboratory. The sampling procedure was repeated every 3 weeks for the entire finishing period (March to June 2007). The samples were cultured for E. coli O157:H7 and Salmonella.

Laboratory methods. Fecal samples were cultured for E. coli O157:H7 as previously described (12). For each sample, 10-g fecal sample was placed in 90 ml of gram-negative enrichment broth containing cefixime-tellurite (0.05 μg/ml) and potassium tellurite (2.5 μg/ml) (Dynal Biotech ASA) and incubated for 6 h at 37°C. One milliliter of this culture was subjected to O157 immunomagnetic separation as described by the manufacturer (Dynal Biotech ASA). Twenty micro liters of the bead-bacteria mixture was plated on sorbitol-MacConkey agar (Difco, Becton Dickinson) plates containing cefixime (0.05 μg/ml) and potassium tellurite (2.5 μg/ml) (CT-SMAC; Dynal Biotech ASA) and incubated overnight at 37°C. Individual sorbitol-nonfermenting colonies were subcultured for isolation on CT-SMAC (Dynal Biotech ASA) plates, and an individual sorbitol-nonfermenting colony from each plate was inoculated onto both MacConkey and Fluorocult agars (Difco, Becton Dickinson). Isolates that fermented lactose but not sorbitol within 24 h and had a negative 4-methylumbelliferyl-β-D-glucuronide (MUG) (Difco, Becton Dickinson) reaction were tested for E. coli O157 and H7 antigens by latex agglutination (Remel, Lenexa, Kans.). Similarly, the RAJ mucosal swabs were vortexed for 1 min, followed by a 6-h incubation as described previously (12). One milliliter of this culture was subjected to O157 immunomagnetic separation (Dynal Biotech ASA) as was performed for the fecal samples. A similar cultural and isolation procedure was performed for all the RAJ mucosal swab samples. Positive isolates for O157 antigen by latex agglutination were further tested in two primer-pair multiplex PCR assay that detected genes for Shiga-like toxins 1 (Stx1) (21) and 2 (Stx2) (20). Positive PCR results for E. coli O157:H7 indicated a detection of genes for the somatic antigen O157, the flagella antigen H7, and at least one or both Shiga toxins (Stx1 or Stx2).

For Salmonella, the samples were cultured in the laboratory using culture methods optimized for the detection of Salmonella (14). Briefly, a sterile swab was loaded with fecal sample and preenriched in buffered peptone water (Difco, Becton Dickinson) at 37°C overnight followed by immunomagnetic beads separation specific for Salmonella species (Dynabeads anti-Salmonella, Dynal Biotech, Inc., Lake Success, N.Y.) according to the manufacturer’s instructions. After the final wash, the beads were transferred to 10 ml of Rappaport Vassiliadis R10 (RV) broth (Becton Dickinson) and incubated (with constant gentle shaking) at 42°C for 24 h. Following incubation, the RV cultures were streaked onto modified brilliant green agar (Becton Dickinson) and mannitol lysine crystal violet brilliant green agar (Oxoid, Basingstoke, UK). Colonies with typical Salmonella characteristics (11) were stabbed in 10-ml triple sugar iron agar slants (Becton Dickinson), and the biochemical results read after 24-h incubation as described (11). Presumptive-positive isolates were sent for serotyping to the National Veterinary Services Laboratories (NVSL) (Ames, Iowa).

Statistical methods. Epi Info, version 3.3.2 (Centers for Disease Control and Prevention, Atlanta, Ga.), was used for statistical analyses. The prevalence of E. coli O157:H7 and Salmonella among the treatment and the control groups were compared and statistical significance of the difference assessed at an α set at ≤0.05. The Fisher’s exact test was used to assess the difference where there were <5 values in a cell. The effects of pen, block, and sampling times on the recovery E. coli O157:H7 and Salmonella were investigated. Least-square means of parameter estimates from the binomial logistic regression was used to estimate adjusted probabilities of fixed effects of treatment, pen, block, and
sampling time. The odds ratio for the recovery of \textit{E. coli} O157: H7 and \textit{Salmonella} in both the treatment and control groups through the sampling periods were calculated.

RESULTS

\textit{E. coli} O157:H7. The baseline information on the percentage of pretreatment fecal shedding of \textit{E. coli} O157:H7 in feces among the two selected group of steers were obtained. Treatment (43.9%) and control (44.4%) pens exhibited no statistically significant differences (\(P = 0.54\)) (Fig. 1). In total, \textit{E. coli} O157:H7 was isolated from 194 (42.4%) of 458 fecal samples. Overall, the block had no effect within treatment groups (\(P = 0.39\)) on the recovery of \textit{E. coli} O157:H7 from the steers, whereas pen demonstrated a significant difference in \textit{E. coli} O157:H7 recovery (\(P = 0.01\)). There was no interaction between DFM treatment and sampling time (\(P = 0.99\)). However, sampling time significantly influenced \textit{E. coli} O157:H7 shedding (\(P = 0.0081\)). Two steers of 138 were taken out of the study due to ill health after the second sampling period. Also, in the second sampling corresponding to the week 3 of DFM treatment, a significant difference (\(P = 0.001\)) in the proportion of steers shedding \textit{E. coli} O157:H7 in treatment groups (16 of 66, 24.2%) and control groups (48 of 71, 67.6%) were calculated (Fig. 1). Similarly, a significant difference (\(P = 0.003\)) in the proportions of steers shedding \textit{E. coli} O157:H7 was recorded at third sampling (week 6), in 29% (\(n = 18\) of 66) treatment and 55.1% (\(n = 38\) of 71) control groups. In the final sampling (week 9 of DFM treatment), only 46 steers were sampled; the rest had been taken for slaughter earlier in the week. Of those sampled, 43.5% (10 of 23) and 13% (\(n = 3\) of 23) of steers in the control and treatment groups, respectively, were positive for \textit{E. coli} O157:H7. This difference in shedding demonstrated statistically significant differences (\(P = 0.01\)). The overall probability of recovery of \textit{E. coli} O157:H7 from the feces of the treatment and the control steers was 34.0% (66 of 221) and 66.0% (128 of 237), respectively (\(P < 0.001\)) (Fig. 1). Overall, supplementing steers with DFM (\textit{L. acidophilus} [LA 51] and \textit{P. freudenreichii} [PF 24]) was associated with a reduction in the natural shedding of \textit{E. coli} O157:H7 from feces (odds ratio, 0.36; 95% confidence interval, 0.25 to 0.53; \(P < 0.001\)).

\textit{Salmonella}. During the pretreatment sampling, 8.3% (6 of 72) of the fecal samples collected from the steers in the control group and 7.6% (5 of 66) in the group selected for DFM treatment were positive for \textit{Salmonella} (\(P = 0.56\)) (Fig. 2). An increase in the shedding of \textit{Salmonella} in both groups was recorded during the second sampling (week 3), although no statistical difference (\(P = 0.355\)) was recorded in the control (18.1%, 13 of 72) and DFM-treated (12.1%, 8 of 66) groups (Fig. 1). Similar but not statistically different trends (\(P = 0.17\)) were observed in the third sampling (week 6) with \textit{Salmonella} prevalence of 20.3 and 17.7% in the control and treatment steers, respectively (Fig. 1). At the last sampling (week 9), \textit{Salmonella} was isolated from 3.7% (1 of 27) of steers on DFM supplement, whereas none (0 of 18) was isolated from the control group.

Overall, \textit{Salmonella} was isolated from 58 of 458 (12.7%) fecal samples tested. All \textit{Salmonella} belonged to the Typhimurium serotype and the majority (53 of 58, 91%) was Typhimurium Copenhagen. Sampling time was shown to have an effect on the recovery of \textit{Salmonella} (\(P = 0.004\)), whereas block (\(P = 0.33\)) and pen (\(P = 0.79\)) did not. The overall probability of recovery of \textit{Salmonella} from the feces of the control (14.0%) and the treated (11.3%) steers were similar (odds ratio, 0.79; 95% confidence interval, 0.46 to 1.37; \(P = 0.24\)).

\textit{E. coli} O157:H7 and \textit{Salmonella} trends. A comparison of the trends in the shedding status of \textit{E. coli} O157:
FIGURE 2. Trends in the shedding status of naturally infected steers with *E. coli* O157:H7 and *Salmonella* over the feeding periods.

H7 and *Salmonella* between sampling times in the infected steers is shown in Figure 2. Analysis of the shedding trends of *E. coli* O157:H7 among the four categories of infected steers (those that maintained shedding, recovered shedders, new shedders, and noninfected steers), showed a significant difference (*P* = 0.05) between DFM-treated (13.8%) and control steers (26.8%) regarding recovered shedders. This trend of a higher recovery rate among untreated steers compared with those fed DFM was seen for both *E. coli* O157: H7 and *Salmonella* all the time, although the difference was not always statistically significant. Once infected, DFM did not improve the probability of recovery from both *E. coli* O157:H7 and *Salmonella* for those steers that were fed DFM compared with their control counterparts (Fig. 2). For *Salmonella*, the rate of new shedders (new infection) was significantly higher (*P* = 0.001) in the controls (21.1%) than the treatment group (7.7%) during week 6 (Fig. 2). Also, the rate of new infections for *E. coli* O157:H7 was observed to be higher among control steers (39.4%) compared with DFM-treated steers (30.8%) during the first 3 weeks, although this difference was not statistically significant. Throughout the study, there was a higher percentage of steers that remained uninfected to both *Salmonella* and *E. coli* O157:H7 in the DFM-treated steers than controls, although these differences were not statistically significant (Fig. 2). Additionally, the odds of detecting *E. coli* O157: H7 was 5.2 times greater than that of detecting *Salmonella* from the control steers (odds ratio, 5.24; 95% confidence interval, 2.6 to 10.5; *P* < 0.001). The recovery of both *Salmonella* and *E. coli* O157:H7 from the 458 fecal samples collected from both treatment and control groups, also differed significantly (*P* < 0.001).

**DISCUSSION**

The prevalence (44.2%) of *E. coli* O157:H7 in naturally infected steers in the pretreatment period was similar to 53.1% reported in a previous study (13). In subsequent sampling times, the shedding of *E. coli* O157:H7 among the control group was higher than in the DFM-treated group. This is consistent with the reports of previous studies (2, 28) in which the prevalence of *E. coli* O157:H7 was shown to be significantly higher among the control steers than their treated counterparts. Also, in this study, pen had a significant effect on *E. coli* O157:H7 shedding in the steers as reported in earlier studies (15). The pen effect has been attributed to the condition of the pen that helps facilitates transmission of *E. coli* O157:H7 via the fecal-oral route between steers (15) and to the presence of high shedders within the pen (16).

Overall, there was a 32% reduction in detectable levels of *E. coli* O157:H7 shed in feces of the steers placed on DFM compared with controls. This report agrees with the 35% reduction in *E. coli* O157:H7 prevalence observed in similar studies by Peterson et al. (22), but lower than the 49 to 57% reduction reported by other researchers (2, 28). The differences in results observed are not fully understood; it could possibly be attributed to the difference in strains used in other studies (*L. acidophilus* [strains NP 45...
and NP 51). The results of this study suggest that the feeding of L. acidophilus (LA 51) and P. freudenreichii (PF 24) to naturally infected cattle with the numbers of CFUs used in this study will significantly decrease, but not eliminate, natural fecal shedding of E. coli O157:H7.

The shedding of Salmonella in cattle has been reported to be unpredictable and to occur in clusters (27). However, in our study, we observed an increasing trend in Salmonella prevalence from the initial pretreatment prevalence of 8.3% and 7.6% to 20.3 and 17.7% in the control and treatment groups, respectively, although those comparisons were not different between the two treatments. Other researchers have reported either no effect (7) or a decrease (25) in Salmonella prevalence in the feedlot over time. This difference in results could be related to the reported erratic nature of Salmonella shedding in cattle (26) or to lack of effect of DFM used in our study. Also, concentration of cattle in the feedlot may facilitate the fecal-oral transmission of enteric bacteria like Salmonella, which may explain the observed increase in Salmonella shedding in our study.

Interestingly, the DFM treatment of steers did not show a beneficial effect on reduction of Salmonella shedding over the study period. In fact, overall, there was a steady increase in Salmonella shedding over the study period in both controls and treatment cattle. The DFM-treated steers were only 1.3 less likely to shed Salmonella in feces than were untreated counterparts in the control (odds ratio, 0.79; 95% confidence interval, 0.46 to 1.37; \( P = 0.24 \)). This is an indication that it would not be economically justifiable to use these strains of DFM as applied in this study as a control strategy for reducing Salmonella shedding in feedlot cattle. The incremental increase of Salmonella shedding in both control and treatment groups warrants further investigations into other DFM combinations or alternative strategies for control of Salmonella infection. Contrary to this study, a recent study by Stephens et al. (26), using strains of L. acidophilus NP 51, reported a dose-response–related effect on reduction of Salmonella among steers placed on DFM. Therefore, it is possible that DFM as applied in our study were not at high enough dose to reduce Salmonella shedding among steers.

Interestingly, our study showed that for Salmonella the rate of new shedders (new infection) was significantly higher (\( P = 0.023 \)) in the controls (21.1%) than the treatment group (7.7%) after 6 weeks on feed. This implies that the DFM were having some protective effect against infection with Salmonella among the steers that were not yet infected. This protective effect against new infections was also observed for E. coli O157:H7 among steers during the first 3 weeks on feed (controls, 39.4%; DFM-treated steers, 30.8%), although the difference was not statistically significant. Also, throughout the study, there was a higher percentage of steers that remained uninfected to both Salmonella and E. coli O157:H7 in the DFM-treated steers than controls, although these differences were not statistically significant. This result may support our hypothesis that, for steers that are not yet infected, DFM may protect them from infection to both Salmonella and E. coli O157:H7. This calls for feeding DFM to steers sooner than later on arrival at the feedlot when fecal shedding of both Salmonella and E. coli O157:H7 are reported to be low (12–14). Previous studies (12–14) have reported E. coli O157:H7 and Salmonella prevalence in steers to be as low as 1.4 and 0.7%, respectively, on arrival at the feedlot. It was evident in our study that more animals were reverting from E. coli O157:H7 shedding to nonshedding status in the control steers (26.8%) than in DFM-fed cattle (13.8%) (\( P < 0.05 \)) during the sixth week on feed. This implies that, for the steers that are already infected with E. coli O157:H7, treating them with DFM may not improve their recovery rate any better than the nontreated steers. This observation underscores the importance of feeding DFM to steers before they are infected with E. coli O157:H7 for maximum benefit. Notably, this trend of a higher recovery rate among nontreated steers compared with those fed DFM was seen for both E. coli O157:H7 and Salmonella all the time, although the difference was not always statistically significant.

It is possible that the observed difference in effect of DFM on E. coli O157:H7 and Salmonella might be related to the difference in mode of action of the DFM for the two organisms. The mechanism of action by which DFM decrease the prevalence of E. coli O157:H7 and other enteric pathogens have been attributed to various phenomena, such as competitive interactions, production of volatile fatty acids, enhancement of specific and total IgA secretion, and secretion of specific antibodies against Stx1, Stx2, and Stx-producing E. coli cells among other effects (19). In a recent study in vitro, the mode of action of L. acidophilus La-5 was shown to be through secretion of molecule(s) that either act as quorum sensing signal inhibitors or directly interact with bacterial transcriptional regulators controlling the transcription of EHEC O157 proteins involved in the colonization of the intestinal tract by EHEC O157:H7 (18). Other normal bacterial floral, some of which are of bovine origin (such as Bacillus circulans), have been reported to inhibit Salmonella enterica serovars Typhimurium DT 104 in vitro (6). Further investigations into more promising alternative strategies for control of Salmonella infection in cattle are warranted. Also, the mechanism for the observed difference in effect of DFM on E. coli O157:H7 and Salmonella warrants further investigation.

The present study provides evidence that DFM as applied in our study were capable of significantly reducing fecal shedding of E. coli O157:H7 in naturally infected cattle but not Salmonella. However, once infected, DFM did not improve the probability of recovery from E. coli O157:H7. Additionally, DFM significantly reduced probability of new infections with Salmonella among DFM-treated cattle compared with controls.

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ERRATA

In the article “Comparative Effect of Direct-Fed Microbials on Fecal Shedding of Escherichia coli O157:H7 and Salmonella in Naturally Infected Feedlot Cattle” by E. S. Tabe, J. Oloya, D. K. Doetkott, M. L. Bauer, P. S. Gibbs, and M. L. Khaitsa, which appears in the Journal of Food Protection 71(3):539–544, the probiotic strains Lactobacillus acidophilus (LA 51) and Propionibacterium freudenreichii (PF 24) were quoted in error. The correct probiotic strain used in the study was Lactobacillus acidophilus (BT 1386).

In the article “Modification of the Submerged Coil To Prevent Microbial Carryover Error in Thermal Death Studies” by S. E. Keller, A. G. Shazer, G. J. Fleischman, S. Chirtel, N. Anderson, and J. Larkin that appears in the Journal of Food Protection 71(4):775–780, in row 1, last column of Table 1, the D-value of Y. pseudotuberculosis in the unmodified submerged coil using a continuous method should be changed from 1.5 ± 0.8 to 15 ± 0.8.