ORIGINAL ARTICLE

Reduced Burden of *Salmonella enterica* in Bovine Subiliac Lymph Nodes Associated with Administration of a Direct-fed Microbial

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Impacts

- *Salmonella* can be harboured within peripheral lymph nodes (PLN) of cattle and, as such, may contribute to *Salmonella* contamination of ground beef products.
- This study was conducted to test the efficacy of *Lactobacillus animalis* (NP51) and *Propionibacterium freudenreichii* (NP24) as a pre-harvest intervention for *Salmonella* when fed as a direct-fed microbial (DFM) in feedlot cattle diets.
- Results from this study illustrate that feeding *Lactobacillus animalis* NP51 and *Propionibacterium freudenreichii* NP24 at 10^9 cfu/head/day as a pre-harvest intervention may aid in reducing *Salmonella* prevalence and concentration in bovine subiliac lymph nodes (SLNs) destined for ground beef.

Keywords:
Salmonella; bovine lymph nodes; direct-fed microbial; beef products; pre-harvest interventions

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Summary

Despite effective food safety interventions within abattoirs, *Salmonella enterica* remains a common contaminant of raw ground beef. Research has recently implicated peripheral lymph nodes (PLNs) as a potential route by which *Salmonella* contaminates ground beef. This study examined the efficacy of using *Lactobacillus animalis* (formerly designated *Lactobacillus acidophilus*; NP51) and *Propionibacterium freudenreichii* (NP24), at 10^9 cfu/head/day, as a direct-fed microbial (DFM) in feedlot cattle diets to control *Salmonella* within PLNs. Two studies were conducted in which cattle were randomly allocated into either control or DFM treatment groups. Diets of treated cattle were supplemented with 10^9 cfu/head/day of the DFM, while control groups received no DFM supplementation. During slaughter at abattoirs, one subiliac lymph node (SLN) per carcass was collected from 627 carcasses from one study and 99 carcasses from the second study. Lymph nodes were cultured to estimate the presence and concentration of *Salmonella*. In the first study, effects of DFM supplementation varied across slaughter days. On the first and second slaughter days, prevalence was reduced by 50% ($P = 0.0072$) and 31% ($P = 0.0093$), respectively. No significant difference was observed on slaughter day three ($P = 0.1766$). In the second study, *Salmonella* was 82% less likely ($P = 0.008$) to be recovered from SLNs of treatment cattle. While a greater relative risk reduction was observed in the latter study, absolute risk reductions were similar across studies. A significant reduction in the concentration of *Salmonella* in SLNs ($P < 0.0001$) on a cfu/g and cfu/node basis was also observed in cattle administered NP51 and NP24 in the first study; in the second study, too few quantifiable SLNs were observed to facilitate meaningful comparisons. The results indicate that NP51 and NP24 supplementation may aid in reducing the prevalence and concentration of *Salmonella* in SLNs and, therefore, serve as an effective control measure to reduce *Salmonella* in ground beef products.

Introduction

Non-typhoidal *Salmonella* is a leading cause of death due to bacterial contamination of food (Scallan et al., 2011). In the United States, *Salmonella enterica* (herein referred to as *Salmonella*) causes an estimated 1.3 million cases of gastroenteritis annually (Scallan et al., 2011). Despite considerable efforts within abattoirs to decrease the burden of this pathogen in the food supply, the human burden of illness caused by this pathogen has persisted over time (CDC, 2008). In some regions, cattle appear to be a natural reservoir for *Salmonella* (Barkocy-Gallagher et al., 2003; Rivera-Betancourt et al., 2004; Kunze et al., 2008); as a consequence, beef products are at risk of contamination. In fact, in 2008, the CDC determined that 1 in 7 *Salmonella* outbreaks, where a specific food vehicle was identified, were attributed to beef (CDC, 2008; Brichta-Harhay et al., 2012). However, estimates of the prevalence of *Salmonella* among feedlot cattle and those cattle presented at abattoirs are quite variable (Barkocy-Gallagher et al., 2003). Variability can also be observed in the literature among studies examining the prevalence in commercial ground beef with some studies having reported estimates as low as 0.42% and others having reported recovering *Salmonella* from up to 4.2% of ground beef samples (Bosilevac et al., 2009; Koohmaraie et al., 2012; Vipham et al., 2012). Differences in methodologies and reported estimates make it difficult to create meaningful comparisons between published data sets. Nevertheless, results from these studies indicate that, despite the development and implementation of effective food safety interventions that reduce carcass contamination, *Salmonella* in beef products is still a concern.

The presence of *Salmonella* within cattle peripheral lymph nodes (PLN) has been well documented (Arthur et al., 2008; Brichta-Harhay et al., 2012; Haneklaus et al., 2012; Koohmaraie et al., 2012; Gragg et al., 2013a,b). Because the complete removal of PLNs from carcasses is not practically possible, PLNs are commonly integrated into ground beef during processing (Koohmaraie et al., 2012). Current interventions applied at the abattoir only reduce surface pathogens; *Salmonella* that is internalized within the PLN are protected from post-harvest interventions that reduce pathogens on the carcass surface only. Therefore, *Salmonella* contained within PLNs likely contribute to ground beef contamination (Arthur et al., 2008; Brichta-Harhay et al., 2012; Koohmaraie et al., 2012; Gragg et al., 2013a).

Pre-harvest interventions are needed to reduce *Salmonella* carriage within PLNs prior to slaughter. While published data are sparse concerning pre-harvest control of *Salmonella*, supplementation of cattle diets with *Lactobacillus animalis* (formerly designated *Lactobacillus acidophilus*; NP51) and *Propionibacterium freudenreichii* (NP24) as a direct-fed microbial (DFM) may hold promise for the reduction of multiple pathogens. In particular, NP51 has been associated with reduced faecal shedding of *E. coli* O157:H7 (Brashears et al., 2002, 2003; Elam et al., 2003; Younts-Dahl et al., 2004, 2005; Loneragan and Brashears, 2005; Peterson et al., 2007). Similar reductions have been observed for *Salmonella*, with one study reporting that *Salmonella* was also less likely to be recovered from the hides or faeces of cattle supplemented with NP51 (Stephens et al., 2007). While studies vary in the concentration of NP51 administered to the animal, a dose of 10^9 cfu/head/day appears to be the most effective dose in reducing pathogens in the animal as a pre-harvest intervention (Younts-Dahl et al., 2005). The objective of this study was to determine whether the administration of NP51 and NP24 (Bovamine® Defend™) – at a dose of 10^9 cfu/head/day – would result in a reduced burden of *Salmonella* in subiliac lymph nodes (SLNs) of cattle presented for slaughter for human consumption.

Materials and Methods

To meet our objectives, two studies were conducted. The first study was conducted in a commercial feedlot setting with pens of approximately 75 animals each. The second study was conducted in a research feedlot at Texas Tech University (Institutional Animal Care and Use Committee (IACUC) protocol 12033-04) pens of approximately 4 animals each.

Background on cattle

Steers for the first study were predominantly of British breeding, whereas steers of the second study were crossbred cattle with varying amounts of *Bos indicus* influence. Commercial steers were located at a feedlot in the Panhandle of Texas, while the research feedlot steers were housed at the Texas Tech University Burnett Center. Arrival weights were recorded, and standard health treatments and feeding routines were followed as specified by protocols at the commercial feedlot and the research feedlot.

Pen assignments

For the commercial feedlot study, approximately 1800 steers were allocated to 12 blocks. Within each block, animals were randomly allocated to the treatment or control cohort with approximately 75 animals/pen. The cattle were slaughtered in three groups and therefore housed at the feedlot for 129, 142 and 151 days, respectively.

In the research feedlot study, a total of 112 steers were blocked by weight and randomly allocated within block

into either the treatment or the control cohort with approximately four steers per pen. The cattle were housed at the research feedlot for 117 days.

Commercial feedlot treatment diets

Commercial feedlot cattle received a starter diet and a finishing diet during the feeding period. The treatment diet differed from the control cattle diet by the addition of NP51 and NP24 (Bovamine® Defend™; Nutrition Physiology Company, LLC (NPC, Guymon, OK, USA)) according to the manufacturer's recommendation, with the target dose being 10^9 cfu/head/day. Treatment and control diets were administered for the duration of the feeding period, and separate feeding trucks were used to administer the two different diets.

On a dry matter basis, the diet consisted of steam-flaked corn (62.0%), wet corn gluten feed based (12.0%, Sweet Bran®, Cargill Corn Milling, Dalhart, TX, USA), dry corn distiller's grains (8.1%), triticale silage (7.0%, fed for first 15 days), corn silage (8.9%, fed for the remainder of the feeding period), fat: animal blend (1.4%) and a dry meal supplement (3.3%, Cargill Animal Nutrition, Guymon, OK, USA). A blend of condensed corn distiller's solubles: glycerine:cane molasses:water (50:30:12:8) made up 4.2% of the diet, on a dry matter basis, and was fed for the first 10 days. For the remainder of the feeding period, a blend of condensed corn distiller's solubles:glycerine (70:30) was fed (Table 1).

Cattle also received monensin (37 mg/kg, Rumensin®, Elanco Animal Health, Greenfield, IN, USA), tylosin (10 mg/kg, Tylan®, Elanco Animal Health) and zilpaterol hydrochloride (8.4 mg/kg, Zilmax®, Merck Animal Health, Kenilworth, NJ, USA). Zilpaterol hydrochloride was only included in the diet for the final 20 days with a 3-day withdrawal period prior to harvest.

Table 1. Ingredient composition of the commercial feedlot diet, on a dry matter basis

Ingredient	% of diet, dry matter basis (DM)
Corn grain, steam flaked	62.0
Corn gluten feed wet[a]	12.0
Corn distillers grains, dry	8.1
Triticale silage[b]	7.0
Corn silage[b]	8.9
Fat, animal blend	1.4
Commodity liquid[c]	4.2
Supplement[d]	3.3
Total	100.00

[a]Sweet Bran®, Cargill Corn Milling.
[b]Triticale silage fed for the first 15 d; corn silage was fed for the remainder of the study.
[c]A 50:30:12:8 blend of condensed corn distiller's solubles: glycerine: cane molasses: water was fed for the first 10 d, and a 70:30 blend of condensed corn distiller's solubles: glycerine was fed for the remainder of the study.
[d]Dry meal supplement manufactured by Cargill Animal Nutrition.

Research feedlot treatment diets

Treatment and control diets differed by the inclusion of NP51 and NP24 at 10^9 cfu/head/day (Bovamine® Defend™, Nutrition Physiology Company, LLC (NPC)) in the treatment diet. A commercially available freeze-dried powder formulation containing NP51 and NP24 at 10^9 cfu/head/day (Bovamine® Defend™, Nutrition Physiology Company, LLC (NPC)) was manually added to the treatment diet ration following the manufacturer's instructions and mixed by the feed trucks. The product was added to the feed after the feed was loaded into the truck and was administered for the duration of the study. Cattle were fed in bunks twice a day and the use of separate feed trucks prevented cross-contamination between the control and the treatment diets.

On a dry matter basis, the feed ration consisted of steam-flaked corn (58.2%), wet corn gluten feed based (24.98%, Sweet Bran®), alfalfa hay (10.06%), tallow (3.00%), urea (0.42%), limestone (1.35%) and supplement (2.00%). The supplement included 29.9 mg/kg of monensin (Rumensin, Elanco Animal Health), 10.0 mg/kg of tylosin (Tylan® Premix, Elanco Animal Health). Ractopamine hydrochloride (Optaflexx®, Elanco Animal Health) at a level of 200 mg/head/d was included in the feed rations during the last 30 days of the feeding period (Table 2).

Sample collection and analysis

During the feeding and SLN sample collection, a code was created and assigned to each pen. Investigators and

Table 2. Ingredient composition of the research feedlot diet, on a dry matter basis

Ingredient	% of diet, dry matter basis (DM)
Corn grain, steam flaked	58.20
Alfalfa hay, mid bloom	10.06
Tallow	3.00
Urea	0.42
Limestone	1.35
TTU-2.0 Supplement[a]	2.00
Sweet bran[b]	24.98
Total	100.00

[a]TTU-2.0 supplement included 29.9 mg/kg of monensin (Rumensin, Elanco Animal Health) 10.0 mg/kg of tylosin (Tylan® Premix, Elanco Animal Health). Ractopamine hydrochloride (Optaflexx®, Elanco Animal Health) at a level of 200 mg/head/day fed for the last 30 day.
[b]Sweet Bran®, Cargill Corn Milling.

individuals involved in sample collection and processing were blinded using this coding system (e.g. rep 1 treatment pen was coded as M10 and control was M11). Codes were lifted after data collection to conduct statistical analysis. In the commercial feedlot study, one SLN per carcass was collected from a convenience sample of an average of 25 animals/pen immediately after slaughter ($n = 627$). In the research feedlot study, SLNs were collected from each animal immediately after slaughter ($n = 99$). All SLNs collected for both the commercial and the research feedlot studies were placed on ice following collection and transported to the laboratory at Texas Tech within 6–8 h for qualitative and quantitative *Salmonella* analyses.

Lymph node processing

As described previously (Brichta-Harhay et al., 2012; Gragg et al., 2013b), visible fat and fascia were trimmed from SLNs, and the weight of each trimmed node was recorded. Nodes were then immersed in boiling water for 3–5 s, placed into plastic bags (24 oz. filter Whirl-pak bags, Nasco, Fort Atkinson, WI, USA) and pulverized using a rubber mallet. Samples were subsequently homogenized by adding 80 ml of trypticase soy broth (TSB) to each bag and stomached (model 400 stomacher, Seward, Worthington, UK) at 230 rpm for 2 min. For qualitative culture purposes, homogenates were incubated at room temperature for 2 h and then at 42°C for 12 h. Immunomagnetic separation (IMS) was conducted using anti-*Salmonella* paramagnetic beads (Dynabeads, Invitrogen, Oslo, Norway) and an automated bead retriever (Dynabead Retriever) according to the manufacturer's instructions. One hundred microlitres of the bead-bacteria suspension was added to 3 ml of Rappaport–Vassiliadis (RV) (EMD Chemicals, Gibbstown, NJ, USA) broth and incubated at 42°C for 18–20 h. Enrichments were streaked onto brilliant green sulfa (BGS) (BD, Difco Laboratories; Becton, Dickinson & co., Sparks, MD, USA) and xylose desoxycholate (XLD) (EMD Chemicals) agar plates. Characteristic colonies of *Salmonella* on at least one type of agar were considered presumptive positives. Commercial agglutination kits (Oxoid, Thermo Scientific, Remel Inc., Lenexa, KS, USA) were used for further identification of morphologically typical colonies.

Salmonella enumeration

Quantitative culture methods were conducted as described by Gragg et al. (2013a) in that 1 ml of the TSB/SLN homogenate was removed prior to initial incubation, plated in duplicate onto *Enterobacteriaceae* count plates (EB; Petrifilm™, 3M, St Paul, MN, USA) and incubated for 22–26 h at 37°C. Colonies were counted according to the manufacturer's instructions and recorded. Bacterial growth on EB count plates (petrifilm™) was transferred to XLD agar and incubated for 16 h at 37°C. Morphologically typical colonies on XLD plates were counted, and comparisons were made with EB count plate (petrifilm™) counts. Concentrations of *Salmonella* were reported on a cfu/g and cfu/node basis.

Statistical analyses

For qualitative data, r/n (events/trials) binomial response variables were created for each pen where r is the number of positives and n is the number of nodes assayed. Generalized linear mixed models using a log-link function were constructed, in which block was considered a random variable. Model estimation was achieved using a residual-based pseudo-likelihood method, and denominator degrees of freedom were estimated using Kenward–Roger calculations. To account for potential within pen dependency among residuals (i.e. clustered outcomes), an overdispersion term was forced into the model. The least square means for the treatment by time interaction were separated using pairwise differences for the prevalence data analysis.

Concentration data were $\log_{10}$-transformed and analysed using a mixed linear model. Concentration analysis was only performed on positive samples. In instances where a sample was positive but could not be enumerated (i.e. above the limit of detection but below the limit of quantification), a fixed value of 1 and 20 was included for cfu/g and cfu/node concentrations, respectively. These values were calculated using half of the limit of quantification for EB count plates. To obtain logcfu/g and logcfu/node, the above values were $\log_{10}$-transformed and yielded values of 0 and 1.3, respectively. Due to the low number of quantitated observations in the research feedlot study, formal statistical analysis was not performed.

Sample size was calculated to be sufficient to detect a difference at $\alpha = 0.10$. As such, this α was used in the analysis to detect differences between fixed effects. Least square means were separated using pairwise differences for statistically significant interactions. Model-adjusted relative risks (RR), relative risk reductions, absolute risk reduction (AR) and 95% confidence intervals (C.I. 95%) were calculated. Diagnostics for residuals for each model were conducted using AIC fit statistics, which were obtained using ODS graphics and residual panel commands. Data were analysed using the SAS System version 9.3 (The SAS Institute, Cary, NC, USA).

Results

Commercial feedlot

The total number of SLNs collected was $n = 627$; of the total number, 310 were collected from cattle administered

NP51 and NP24 (treatment) and 317 were collected from cattle in the control group (Table 3). A total of 176 SLNs collected from cattle in the treatment group tested positive. A greater number of positives (240) were observed in SLNs collected from cattle in the control group (Table 3). On a percentage basis for least square means estimates, the amount of positive SLNs collected from cattle in the treatment group was 57.5%. A higher percentage of positives (76.3%) were observed in SLNs collected from cattle in the control group (Table 4). Variations in positive SLNs by pen level were observed, with pens in the treatment group having as few as 6 positives (27.3%) in a pen and as many as 21 positives (80.8%) in a single pen (Table 3). Less variation was observed among pens in the control group with the average number of positive SLNs being 20; however, one pen did have as few as 10 positive SLNs from 17 collected SLNs (58.8% pen level prevalence). The pen with the highest levels had 27 positive SLNs from 32 collected SLNs (84.3% pen level prevalence), and no pens had 0% or 100% positive SLNs (Table 3).

Table 3. Total number of cattle per pen, total number of subiliac lymph nodes (LNs), total number of positive lymph nodes and *Salmonella* concentrations on a cfu/gram and cfu/node basis for both treatment and control groups for the research feedlot study

	Commercial feedlot study				
Rep	# of cattle/pen	# of subiliac LNs collected	# of positives	Log cfu/gram	Log cfu/node
Treatment group					
1	74	28	11	0.56	1.85
2	75	25	10	0.43	1.77
3	75	24	8	0.68	1.99
4	75	22	6	0.71	2.04
5	74	23	16	1.41	2.77
6	73	31	20	1.29	2.62
7	75	27	12	1.18	2.53
8	75	26	20	2.00	3.34
9	75	26	21	2.58	3.95
10	75	26	16	2.53	3.86
11	75	26	18	1.76	3.07
12	75	26	18	1.36	2.66
Total	896	310	176	N/A	N/A
Control group					
1	75	17	10	1.44	2.78
2	75	31	24	1.57	2.86
3	75	28	19	2.34	3.67
4	75	27	15	2.23	3.61
5	75	21	17	1.42	2.78
6	74	32	27	1.52	2.84
7	75	29	24	1.67	2.97
8	75	26	24	3.79	5.14
9	75	27	17	2.88	4.22
10	75	26	20	2.60	3.90
11	75	26	21	2.96	4.27
12	74	27	22	2.47	3.72
Total	898	317	240	N/A	N/A

A significant reduction in *Salmonella* prevalence in SLNs was observed from cattle administered NP51 and NP24; however, the effect varied across slaughter days ($P = 0.038$). A relative risk reduction of 50% (RR: 0.50, 95% CL = 0.32, 0.78; $P = 0.007$) and 31% (RR: 0.69, 95% CL = 0.54, 0.89; $P = 0.009$) was observed in *Salmonella* prevalence on the first and second slaughter dates, respectively. However, no significant difference was observed on the third slaughter day (RR: 0.90, 95% CL = 0.76, 1.06; $P = 0.177$). At the time of sample collection, cattle were on feed 129, 142 and 151 days for slaughter days one, two and three, respectively. The total number of SLNs collected in each slaughter day (1, 2 and 3) was $n = 157$, $n = 208$ and $n = 262$, respectively.

On a percentage basis for least square means estimates for slaughter day one, a lower number of positive SLNs were collected from the treatment cattle (33.8%) in comparison with the control cattle (67.8%) (Fig. 1). A similar observation was made in slaughter day two with a reduced number of positive SLNs being collected from treatment cattle (54.6%) in comparison with control cattle (78.7%) (Fig. 1). For slaughter day three, the number of positive SLNs was similar between treatment (71.4%) and control cattle (79.6%) (Fig. 1).

Research feedlot

The total number of SLNs collected was $n = 99$ with 52 collected from cattle administered NP51 and NP24 (treatment) and 47 collected from cattle in the control group (Table 5). The total number of SLNs collected from cattle in the treatment group that tested positive was 3, while the total number of SLNs that tested positive from the control group was 14 (Table 5). On a percentage basis for least square means estimates, 25.9% (Fig. 2) of SLNs collected from control cattle tested positive for *Salmonella,* while only 4.7% (Fig. 2) of SLNs collected from the treatment group tested positive (Table 4).

Salmonella prevalence in bovine SLNs was significantly reduced in cattle supplemented with NP51 and NP24 in the research feedlot study. The relative risk reduction showed that *Salmonella* was 82% less likely (RR = 0.18, 95% CL = 0.07, 0.50; $P = 0.008$) to be recovered from the SLNs of cattle administered NP51 and NP24, compared to control cattle (Fig. 2).

Concentration data

The commercial and research feedlot studies also demonstrated a shift in the concentration of *Salmonella* in SLNs

Table 4. Model-adjusted means, relative risk and risk difference (absolute risk) values for both research feedlot study and commercial feedlot study. Values in parenthesis represent 95% confidence intervals

	Research feedlot study	Commercial feedlot study		
	Single slaughter day	Day 1	Day 2	Day 3
Model adjusted mean (control)	0.259 (0.109, 0.665)	0.678 (0.562, 0.818)	0.787 (0.677, 0.915)	0.796 (0.700, 0.906)
Model adjusted mean (treatment)	0.047 (0.016, 0.137)	0.338 (0.228, 0.500)	0.546 (0.443, 0.672)	0.714 (0.620, 0.823)
Relative risk	0.182 (0.066, 0.512)	0.50 (0.32,0.78)	0.69 (0.54, 0.89)	0.90 (0.76,0.94)
Risk difference (absolute risk)	0.24	0.34	0.25	0.07

due to the influence of supplementation of NP51 and NP24. Higher concentrations of *Salmonella* were observed in SLNs from cattle in the control group than in those collected from cattle that had been supplemented with NP51 and NP24. It is important to note that due to enumeration data being sparse for the research feedlot study, no statistical data analysis was conducted.

No significant interaction was observed between concentration and slaughter day on a cfu/g and cfu/node basis ($P = 0.152$ and $P = 0.176$, respectively) in the commercial study. Averaged across day, a significant reduction in the concentration on a $\log_{10}$ cfu/g and $\log_{10}$ cfu/node basis was observed ($P = <0.001$ for both per g and per node; Fig. 3) among treated cattle (1.45 [95% CL = 1.03, 1.87] and 2.78 [95% CL = 2.36, 3.20], respectively) compared to the control animals (2.25 [95% CL = 1.85, 2.65] and 3.57 [95% CL = 3.17, 3.97], respectively). An approximate average of nearly a 1-log difference in the concentration of *Salmonella* on a cfu/g and cfu/node basis on the pen level between the treatment and the control groups was observed (Table 3).

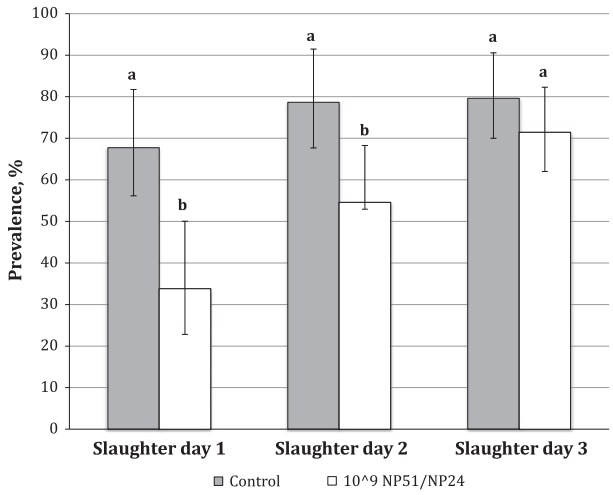

Fig. 1. Model adjusted prevalence of *Salmonella* in subiliac lymph nodes in commercial feedlot for control cattle and cattle fed 10^9 CFU/head/day of *Lactobacillus animalis* (NP51) and *Propionibacterium freudenreichii* (NP24) for each slaughter day. The total number of positives from slaughter day one was 82/157, with total number of 58/86 positives in the control group and 24/71 positives in the treatment group. The total number of positives from slaughter day two was 137/208, with a total number of 78/99 positives in the control group and 59/109 positives in the treatment group. The total number of positives from slaughter day three was 197/262, with 104/132 positives in the control group and 93/130 positives from the treatment group. Bars represent upper and lower model adjusted 95% confidence levels. Columns with different letters are significantly different ($P < 0.10$).

Discussion

In this study, a consistent and absolute reduction in the prevalence of *Salmonella* within SLNs was observed across two studies on cattle presented for harvest destined for human consumption. Moreover, we detected a reduction in the concentration of *Salmonella* harboured in SLNs of the cattle in the commercial feedlot study. The data reported herein indicate that administering NP51 and NP24 to cattle during the feeding period has an effect on the prevalence and concentration of *Salmonella* detected in

Table 5. Total number of cattle per block (two pens per block), total number of subiliac lymph nodes (LNs) and total number of positives lymph nodes for both treatment and control groups for the research feedlot study

	Research feedlot study		
Rep	# of cattle/block	# of subiliac LNs collected	# of positives
Treatment group			
1	7	7	0
2	8	8	1
3	8	8	1
4	8	8	0
5	8	8	0
6	7	7	0
7	6	6	1
Total	52	52	3
Control group			
1	7	7	1
2	7	7	2
3	5	5	4
4	6	6	0
5	6	6	1
6	8	8	3
7	8	8	3
Total	47	47	14

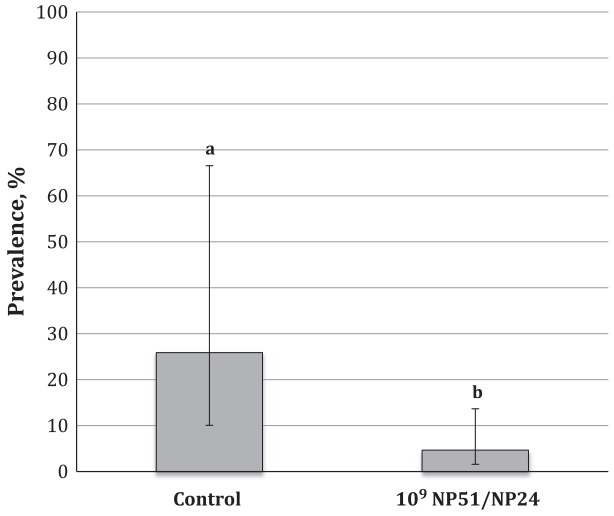

Fig. 2. Model adjusted prevalence of *Salmonella* in subiliac lymph nodes in the research feedlot for control cattle and cattle fed 10^9 CFU/head/day of *Lactobacillus animalis* (NP51) and *Propionibacterium freudenreichii* (NP24). The total number of positives from the research feedlot study was 14/47 from the control group and 3/52 from the treatment group. Bars represent upper and lower model adjusted 95% confidence levels. Columns with different letters are significantly different ($P < 0.10$).

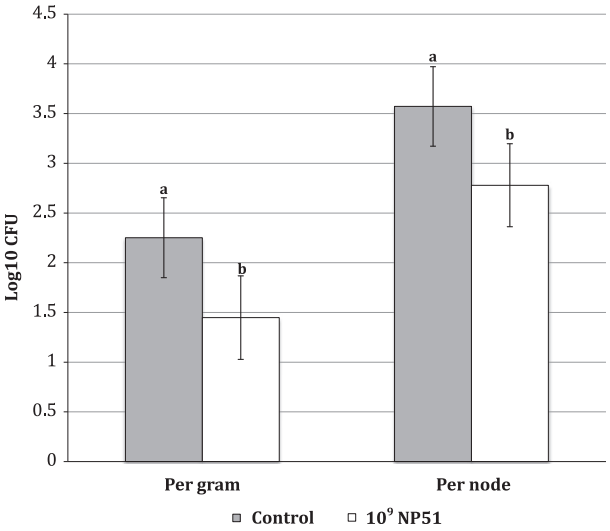

Fig. 3. Concentration of *Salmonella* per gram and per node for control and supplemented cattle for the commercial feedlot study. The total number of quantifiable SLNs from the control group was 191 while the total number of quantifiable SLNs from the treatment group was 150. Bars represent upper and lower 95% confidence levels. Columns with different letters are significantly different ($P < 0.10$).

SLNs of beef cattle. These data are impactful for the beef industry as well as public health, as lymph nodes, including SLNs, are commonly incorporated into beef trim destined for ground beef production.

While the observed reduction varied across sample day in the study of cattle housed in a commercial feedlot, it is tempting to conclude that in general, a greater reduction of *Salmonella* was observed in cattle from the research feedlot study. However, it is important to note that percentage reductions observed are relative (i.e. derived from a relative risk). If absolute differences are considered, the reductions were generally similar across the two studies.

A significant reduction of *Salmonella* in the SLNs of beef cattle was illustrated by our data. Although current research has demonstrated that *Salmonella* can be commonly detected in SLNs, contamination is not limited to the subiliac. Data illustrate that several lymph nodes are capable of harbouring *Salmonella,* and it is important to note the complexity of the bovine lymphatic system in order to fully understand the limitations of our data and the inferences that can be made from it. Further investigations into the ecology of *Salmonella* within the bovine lymphatic system are a goal for future research and will provide a more in-depth understanding of this issue.

Due to the above-stated complexity of the bovine lymphatic system, it is also important to highlight the existence of multiple lymph nodes of the same type. The bovine lymphatic system includes two SLNs; however, in our study, only one SLN was collected, allowing for the possibility that the uncollected lymph node potentially may or may not harbour *Salmonella.* This may be problematic when making inferences from the data stated herein, due to the fact that these data only support *Salmonella* prevalence on a SLN level and not on a carcass level. However, much of the body of work on *Salmonella* prevalence in bovine lymph nodes is comprised of studies that examined only one lymph node (Arthur et al., 2008; Haneklaus et al., 2012; Koohmaraie et al., 2012; Gragg et al., 2013a,b). Furthermore, Gragg et al. (2013b) state that the SLN is the second most commonly contaminated lymph node in the bovine system, with a prevalence of 76.5%. This may lend to a smaller likelihood that reductions observed in this study are due to the chance that the negative lymph node was collected. Additionally, upon observation of the data presented in Table 3 and 5, the biological differences between positive samples among control and treatment groups can be observed on the pen/block level as well as overall. The possibility that these reductions are due to chance and chance alone is present; however, the probability of this occurring does not seem likely. Nevertheless, it is a consideration worth noting while examining these data.

The limited size of the pens used in the research feedlot study is a limiting factor in our ability to make inferences to the U.S. commercial feedlot industry from the data collected. Cattle in the research facility were grouped with approximately four animals to a pen, while cattle in the commercial feedlot were grouped by up to 75 animals in a

pen. Grouping of cattle in the research study is not an accurate depiction of the source population, and it is important when interpreting these data to consider this limitation. Considering the absolute risk reduction between the research study and the commercial feedlot study allows for a better frame of reference for interpretation.

For both the commercial and the research feedlot cattle studies, the overall *Salmonella* prevalence in control cattle SLNs was higher than a majority of what has been reported in the literature. Seasonal as well as regional variables may have contributed to these larger percentages. Cattle were on feed in the summer and fall, and sampling of SLNs was conducted in late August and early October. Seasonal effects have been well documented in the literature, with findings showing a higher presence of *Salmonella* in faeces, on hides, and on pre-evisceration carcasses during the summer and fall months (Barkocy-Gallagher et al., 2003; Rivera-Betancourt et al., 2004; Callaway et al., 2007; Kunze et al., 2008; Loneragan et al., 2011; Gragg et al., 2013b). In addition to conducting this study during the summer and fall, this study was also limited to cattle being fed in the Panhandle and South Plains of West Texas.

Previous research on feedlot cattle has indicated an increase in faecal shedding of *Salmonella* in states located in the southern United States, with feedlots in the southern region having a higher likelihood (OR = 3.23, C.I. 90% 1.57–6.64) of *Salmonella* being detected in faecal samples (Green et al., 2010). This same regional effect has also been observed in *Salmonella* presence in PLNs. Gragg et al. (2013a) collected PLNs from the Beef Industry Food Safety Council (BIFSCO) region 3 (Arizona, New Mexico and Texas) and region 5 (Nebraska, South Dakota, North Dakota, Wisconsin and Minnesota). Results from this study suggest that the region from which PLNs were collected appeared to have an effect on *Salmonella* prevalence in feedlot cattle, with a mean percentage of 19.3% in region 3 and 4.6% in region 5 (Gragg et al., 2013a). Given that feedlots in region 3 market approximated 20–25% of all feedlot cattle in the U.S., the use of a proven *Lactobacillus*-based DFM may hold promise for significant reductions of *Salmonella* in feedlot cattle and correspondingly have an impact on ground beef products (National Agricultural Statistics Service (NASS), 2014).

As mentioned previously, the cattle in the commercial feedlot study were slaughtered in three different groups, which resulted in a different time on feed for each group (129, 142 and 151 days). These slaughter days will be referred to as one, two and three for 129, 142 and 151 days, respectively. A statistically significant interaction was observed between treatment and time on feed for slaughter day one and two; however, no significant difference was observed on slaughter day three. Furthermore, no significant difference was observed for slaughter day and *Salmonella* concentration on a cfu/g and a cfu/node basis.

Although dose response has been addressed in the literature (Younts-Dahl et al., 2005), to our knowledge, there are no data available specifically on the efficacy of DFMs over time. This makes it difficult to create meaningful conclusions as to why an interaction was observed in this study. However, a prior study on DFMs has made note of differences in the prevalence of O157:H7 in faecal samples over time (Brashears et al., 2003). Authors for this study observed a decrease over time in the proportion of faecal samples that tested positive for O157:H7, and theorized that a lower amount of positives were observed at the abattoir due to decreased contamination in the environment. This observation appears to contradict results from this study, which may indicate that contamination in the environment had little effect on the prevalence in this study. With this in mind, it is also important to consider the differences between the studies as far as sample size, sample type and pathogen of interest.

Former hypotheses on potential modes of action for DFMs may also direct our thinking on these interactions. One hypothesis for a potential mode of action is the alteration of host immunity. Krehbiel et al. (2003) stated, 'Bacterial DFMs have been shown to affect innate, humoral, and cellular arms of the immune system'. The authors also indicate that feeding of DFMS, such as *Lactobacillus*, can affect immune response by elevating lymphocyte activity, such as natural killer cells. Demeria et al. (2009) echo this observation by indicating that many strains of *Lactobacillus* are capable of eliciting different immune responses, from enhanced epithelial resistance to increased antibody production. Perhaps, although this is solely speculation, initial exposure to a DFM may assist in mounting a more effective immune response. However, as the duration of exposure is increased, there may be an equilibrium occurring in the animal's microbiota, diminishing the effect of the initial response.

Other potential theories may tilt towards simple differences in time of the month; cattle were slaughtered between 30 August and 21 September 2012. This theory seems less substantial due to the fact that a total of only 22 days separated slaughter day one and slaughter day three, with a lesser span of time between each sequential slaughter day. There was also a larger amount of SLNs collected (n = 262) on the third slaughter day compared to slaughter day one (n = 157) and slaughter day two (n = 208), with a difference as large as 105 SLNs between slaughter day one and three. An upward shift in *Salmonella* prevalence was observed in each sequential slaughter day that parallels an increase in the total number of SLNs collected. There is a possibility that inconsistencies in the number of SLNs collected for each slaughter day had an affect on *Salmonella*

prevalence. Other factors may be structured around changes in weather, pen differences, water, etc. Although these changes may have affected the data during each slaughter day, it may be unwise to draw any conclusions based on these factors, due to the fact that it would be mere speculation. Deeper investigation on the effect of time on the efficacy of DFMs is necessary to have a more complete understanding.

Results from this study indicate that the usage of NP51 and NP24 as a pre-harvest intervention will aid in reducing *Salmonella* prevalence and concentration in bovine SLNs. It is important to note the dose of the product administered to the cattle in this study was 10^9 cfu/head/day, as other products on the market suggest a lower dose (10^7 cfu/head/day). These data show a significant reduction of *Salmonella* in SLNs – not only on a percentage basis but also on a concentration basis – in a commercial feedlot setting, as well as a significant reduction in prevalence in a research feedlot setting. Although the literature reveals a gap in the research available for the effects of DFMs on lymph node contamination, our findings are consistent with the current understanding of the efficacy of NP51 and NP24 in reducing pathogen carriage in ruminant systems. Further, these data support previously cited studies that have shown that feeding NP51 and NP24 reduces pathogen shedding by animals in feedlot settings, thus reducing pathogen loads entering harvest facilities as well as ground beef processing facilities. Successful pre-harvest interventions such as NP51 and NP24 can possibly aid in outbreak prevention, reduce recall costs not only for the industry but also for the government as well, and strengthen protection of public health.

Although the data described herein suggest feeding DFM may aid in reducing the burden of *Salmonella* in SLNs, complete elimination was not observed, and thus, this study also raises further questions. While recent studies have been published on the ecology of *Salmonella* within a bovine system (Gragg et al., 2013a), as well as studies using challenge models to understand the modes in which a PLN becomes contaminated with *Salmonella* (Edrington et al., 2013), gaps still exist. Important directions for future research include the investigation of the routes of entry, the interaction of the pathogen within the bovine immune system, the *in vivo* interaction between pathogen and DFM, bovine immune system and DFM interactions, as well as, the interaction effect between treatment and time.

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References

Arthur, T. M., D. M. Brichta-Harahay, J. M. Bosilevac, M. N. Guerini, N. Kalchayanand, J. E. Wells, S. D. Shackelford, T. L. Wheeler, and M. Koohmaraie, 2008: Prevalence and characterization of *Salmonella* in bovine lymph nodes potentially destined for use in ground beef. *J. Food Prot.* 71, 1685–1688.

Barkocy-Gallagher, G. A., T. M. Arthur, M. Rivera-Betancourt, X. Nou, S. D. Shackelford, T. L. Wheeler, and M. Koohmaraie, 2003: Season prevalence of shiga toxin-producing *Escherichia coli*, including O157:H7 and non-O157 serotypes, and *Salmonella* in commercial beef processing plants. *J. Food Prot.* 66, 1978–1986.

Bosilevac, J. M., M. N. Guerini, N. Kalschayanand, and M. Koohmaraie, 2009: Prevalence and characterization of Salmonellae in commercial ground beef in the United States. *Appl. Environ. Microbiol.* 75, 1892–1900.

Brashears, M. M., M. L. Galyean, G. H. Loneragan, J. E. Mann, and K. Killinger-Mann, 2002: Prevalence of *Escherichia coli* O157:H7 and performance by beef feedlot cattle given *Lactobacillus* direct-fed microbials. *J. Food Prot.* 66, 748–754.

Brashears, M. M., D. Jaroni, and J. Trimble, 2003: Isolation, selection, and characterization of lactic acid bacteria for a competitive exclusion product to reduce shedding of *Escherichia coli* O157:H7 in cattle. *J. Food Prot.* 66, 355–363.

Brichta-Harhay, D. M., T. M. Arthur, J. M. Bosilevac, N. Kalchayanand, J. W. Schmidt, R. Wang, S. D. Shackelford, G. H. Loneragan, and T. L. Wheeler, 2012: Microbial analysis of bovine lymph nodes for the detection of *Salmonella enterica*. *J. Food Prot.* 75, 854–858.

Callaway, T. R., T. S. Edrington, R. C. Anderson, J. A. Byrd, and D. J. Nisbet, 2007: Gastrointestinal microbial ecology and the safety of our food supply as related to *Salmonella*. *J. Anim. Sci.* 86, E163–E172.

Center for Disease Control, 2008: Surveillance for foodborne disease outbreaks-United States. 2008. *MMWR Morb. Mortal. Wkly Rep.* 60, 1197–1202.

Demeria, D., J. Ewaschuk, and K. Madsen, 2009: Interactions of *Lactobacillus* with the immune system. In: Ljungh, A., and T. Wadstrom (eds), Lactobacillus Molecular Biology: From Genomics to Probiotics, pp. 139–148. Caister Academic Press, Norfolk, UK.

Edrington, T. S., G. H. Loneragan, J. Hill, K. J. Genovese, H. He, T. R. Callaway, R. C. Anderson, D. M. Brichta-Harhay, and D. J. Nisbet, 2013: Development of a transdermal *Salmonella* challenge model in calves. *J. Food Prot.* 76, 1255–1258.

Elam, N. A., J. F. Gleghorn, J. D. Rivera, M. L. Galyean, P. J. Defoor, M. M. Brashears, and S. M. Younts-Dahl, 2003: Effects of live cultures of *Lactobacillus acidophilus* (strains NP45 and NP51) and *Propionibacterium freudenreichii* on performance, carcass, and intestinal characteristics, and *Escherichia coli* strain O157 shedding of finishing beef steers. *J. Anim. Sci.* 81, 2686–2698.

Gragg, S. E., G. H. Loneragan, M. M. Brashears, T. M. Arthur, J. M. Bosilevac, N. Kalchayanand, R. Wang, J. W. Schmidt, J. C.

Brooks, S. D. Shackelford, T. L. Wheeler, T. R. Brown, T. S. Edrington, and D. M. Brichta-Harhay, 2013a: Cross-sectional study examining *Salmonella enterica* carriage in subiliac lymph nodes in cull and feedlots cattle at harvest. *Foodborne Pathog. Dis.* 10, 368–374.

Gragg, S. E., G. H. Loneragan, K. K. Nightingale, D. M. Brichta-Harhay, H. Ruiz, J. R. Elder, L. G. Gracia, M. F. Miller, A. Echeverry, R. G. Ramirez Porras, and M. M. Brashears, 2013b: Substantial within-animal diversity of *Salmonella* isolates from lymph nodes, feces, and hides of cattle at slaughter. *Appl. Environ. Microbiol.* 79, 4744–4750.

Green, A. L., D. A. Dargatz, B. A. Wagner, P. J. Fedorka-Cray, S. R. Ladely, and C. A. Kopral, 2010: Analysis of risk factors associated with *Salmonella* spp. isolated from U.S. feedlot cattle. *Foodborne Pathog. Dis.* 7, 825–833.

Haneklaus, A. N., K. B. Harris, D. B. Griffin, T. S. Edrington, L. M. Lucia, and J. W. Savell, 2012: *Salmonella* prevalence in bovine lymph nodes differs among feedyards. *J. Food Prot.* 75, 1131–1133.

Koohmaraie, M., J. A. Scanga, M. J. De La Zerda, B. Koohmaraie, L. Tapay, V. Beskhlenbnaya, T. Mai, K. Greeson, and M. Samadpour, 2012: Tracking the source of *Salmonella* in ground beef produced in nonfed cattle. *J. Food Prot.* 75, 1464–1468.

Krehbiel, C. R., S. R. Rust, G. Zhang, and S. E. Gilliland, 2003: Bacterial direct-fed microbial in ruminant diets: performance response and mode of action. *J. Anim. Sci.* 81, E120–E132.

Kunze, D. J., G. H. Loneragan, T. M. Platt, M. F. Miller, T. E. Besser, M. Koohmaraie, T. Stephens, and M. M. Brashears, 2008: *Salmonella enterica* burden in harvest-ready cattle populations from the southern high plains of the United States. *Appl. Environ. Microbiol.* 74, 345–351.

Loneragan, G. H., and M. M. Brashears, 2005: Pre-harvest interventions to reduce carriage of *E. coli* O157 by harvest-ready feedlot cattle. *Meat Sci.* 71, 72–78.

Loneragan, G. H., D. U. Thomson, R. M. McCarthy, H. E. Webb, A. E. Daniels, T. S. Edrington, D. J. Nisbet, S. J. Trojan, S. C. Rankin, and M. M. Brashears, 2011: *Salmonella* diversity and burden in cows on and culled from dairy farms in the Texas high plains. *Foodborne Pathog. Dis.* 9, 549–555.

National Agricultural Statistics Service (NASS), 2014: U.S. Cattle on Feed. USDA, National Agricultural Statistics Board 2014. Available at: http://usda.mannlib.cornell.edu/usda/current/CattOnFe/CattOnFe-09-19-2014.pdf (accessed on 23 September 2014).

Peterson, R. E., T. J. Klopfenstein, G. E. Erickson, J. Folmer, S. Hinkley, R. A. Moxley, and D. R. Smith, 2007: Effect of *Lactobacillus acidophilus* strain NP51 on *Escherichia coli* O157:H7 fecal shedding and finishing performance in beef feedlot cattle. *J. Food Prot.* 70, 287–291.

Rivera-Betancourt, M., S. D. Shackelford, T. M. Arthur, K. E. Westmoreland, G. Bellinger, M. Rossman, J. O. Reagan, and M. Koohmaraie, 2004: Prevalence of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* in two geographically distant commercial beef processing plants in the United States. *J. Food Prot.* 67, 295–302.

Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin, 2011: Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis.* 17, 7–15.

Stephens, T. P., G. H. Loneragan, E. Karunasena, and M. M. Brashears, 2007: Reduction of *Escherichia coli* O157 and *Salmonella* in feces and on hides of feedlot cattle using various doses of a direct-fed microbial. *J. Food Prot.* 70, 2386–2391.

Vipham, J. L., M. M. Brashears, G. H. Loneragan, A. Echeverry, J. C. Brooks, W. E. Chaney, and M. F. Miller, 2012: *Salmonella* and *Campylobacter* baseline in retail ground beef and whole-muscle cuts purchased during 2010 in the United States. *J. Food Prot.* 75, 2110–2115.

Younts-Dahl, S. M., M. L. Galyean, G. H. Loneragan, N. A. Elam, and M. M. Brashears, 2004: Dietary supplementation with *Lactobacillus*- and *Propionibacterium*-based direct-fed microbials and prevalence of *Escherichia coli* O157 in beef feedlot cattle and on hides at harvest. *J. Food Prot.* 67, 889–893.

Younts-Dahl, S. M., G. D. Osborn, M. L. Galyean, J. D. Rivera, G. H. Loneragan, and M. M. Brashears, 2005: Reduction of *Escherichia coli* O157 in finishing beef cattle by various doses of *Lactobacillus acidophilus* in direct-fed microbials. *J. Food Prot.* 68, 6–10.

