# Assessing *Salmonella enterica* burden and control measures in dairy cattle

by

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# Abstract

*Salmonella enterica* stands as an important pathogen of significant public health concern, given its widespread prevalence and distribution. Its presence has been notably identified not only in the feces and on the hides of cattle but also, of recent paramount importance, within their lymph nodes (LN).

This significant concern surrounding *Salmonella* in cattle forms the basis for **Chapter 1** of this thesis which consists of a comprehensive review of the literature on the epidemiology and microbiological characteristics of *Salmonella* in cattle. This chapter encompasses various aspects, including the prevalence and concentration of *Salmonella* in different cattle samples, the risk factors affecting its prevalence and concentration estimates, and the various intervention strategies established with the aim of mitigating this pathogen in cattle.

**Chapter 2** presents up-to-date information on *Salmonella* prevalence estimates, serotype diversity, and antimicrobial-resistant isolates in cattle LN, as well as the effectiveness of a control measure, across different seasons and multiple regions within the U.S., from an observational study. Briefly, the objectives were 1) to determine whether a larger scale or farm-level use of a commercially available postbiotic product (Nutritek™ (Diamond V, Cedar Rapids, Iowa, U.S.)) compared to not feeding a postbiotic product (i.e., No-postbiotic) was associated with the reduction in the prevalence and concentration of *Salmonella* organisms in the subiliac LN of cull dairy cows, across seasons and regions, harvested in two large U.S. commercial processing plants (**Study 1**), 2) to determine associations between cattle type, other demographic and management characteristics, season, plant and region with prevalence and concentration of *Salmonella* in bovine LN (**Study 1**), and 3) to provide a baseline estimate of the *Salmonella* LN

prevalence and serovar diversity from cull dairy cattle obtained from suppliers in different U.S. regions and seasons (**Study 2**). In collaboration with two commercial processing plants in the Southwestern (SW) and Northeastern (NE) regions of the U.S., cull dairy cattle lots processed in the same week from dairy farms that fed the postbiotic or did not feed the postbiotic (No-postbiotic) were identified and sampled (**Study 1**). For the baseline prevalence study, LN samples from dairy farms supplying cattle to five processing plants in four regions (Upper Midwest, Midwest, Northeast, and Southwest) were collected (**Study 2**). Up to 20 LN were collected from each supplier farm at least once every season, for both studies. Samples were analyzed for *Salmonella* by culture-based and quantitative PCR methods. Isolates were subjected to serotype identification via a classical serotyping and molecular typing, as well as to antimicrobial susceptibility testing using the broth micro-dilution method. Although a numerical reduction was observed, the dairy farm administration of a postbiotic product at pre-harvest was not effective at significantly reducing *Salmonella* prevalence in LN of cull dairy cattle. *Salmonella* LN prevalence only significantly varied by region/processing plant, where the Southwest region showed a higher prevalence than its Northeast counterpart. These findings were consistent with the baseline evaluation of *Salmonella* LN prevalence in cull dairy cattle across different U.S. regions and seasons, where region and season were the main drivers of prevalence. Within Study 1, dominant serotypes included Montevideo, Mbandaka, Muenster, Cerro, Meleagridis, and Anatum. The probability of isolating a *Salmonella* serotype did not significantly vary by feed additive status or region, however, it did vary by season. Between 0 and 34 isolates exhibited resistance to each antimicrobial, with streptomycin and ciprofloxacin showing the highest number of resistant isolates, although resistance to these antimicrobials did not significantly vary by feed additive status, region, or season. There were seven isolates that

were considered multidrug-resistant (MDR); one of the MDR isolates belonged to a sample from a postbiotic supplier from the Northeast region, whereas the remaining six isolates were from No-postbiotic samples, five from the Southwest and one from the Northeast region. In Study 2, dominant serotypes included Montevideo, Muenster, Mbandaka, Anatum, and Cerro. As with Study 1, the probability of isolating a *Salmonella* serotype did not significantly vary by region, but it did vary by season. For each antimicrobial, there were between 0 and 35 isolates that exhibited a resistant phenotype, with streptomycin showing the highest number of resistant isolates, although resistance to streptomycin did not significantly vary by season or region. There were four isolates that were considered MDR; two of the MDR isolates belonged to a sample from the Southwest region, one from the Upper Midwest, and one from the Northeast region.

**Chapter 3**, the discussion chapter, serves as a synthesis of the insights garnered from the preceding chapters. Beyond illustrating the significance of the information presented in chapters 1 and 2 for the beef industry and public health at large, the discussion chapter delves into future directions. It highlights the necessity of acquiring a comprehensive understanding of the routes of entry, infection mechanisms, and the persistent survival of *Salmonella*, particularly within the confines of the LN. This understanding may prove pivotal in shaping effective intervention strategies aimed at mitigating *Salmonella* contamination in beef. Ultimately, these strategies are essential for ensuring food safety and safeguarding public health.

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# Dedication

I dedicate this thesis to Almighty God, whose grace and strength have enabled me to successfully complete this degree program.

# Preface

All chapters were formatted in accordance with the guidelines outlined in the EDTR MS thesis template

# Chapter 1 - Literature Review

## 1.1 *Salmonella*: General epidemiology and public health significance

### 1.1.1 Background

*Salmonella enterica* subspecies *enterica*, often referred to as *Salmonella,* is an important pathogenic bacterium that is of public health concern both in the United States (U.S.) and abroad. Of notable concern is the prevalence of non-typhoidal salmonellosis, a condition primarily associated with the consumption of animal-derived foods including poultry, eggs, pork, and beef (Eng et al., 2015). This microorganism is responsible for a substantial global disease burden, contributing to approximately 550 million annual cases of illness worldwide (WHO, 2018). Within the U.S. alone, this bacterium accounts for an estimated 1.35 million infections, resulting in 26,500 hospitalizations and 420 deaths each year (CDC, 2019). According to the U.S. Department of Agriculture – Economic Research Service (USDA – ERS, 2021), the collective financial impact of foodborne illnesses attributed to *Salmonella* in the U.S. surpasses 4 billion dollars, encompassing medical expenditures, reduced productivity, and mortality costs.

Human exposure to *Salmonella* typically occurs through the consumption of contaminated food and water, as well as direct contact with infected animals, their excreta, or their surroundings (CDC, 2019; Eng et al., 2015). The incubation period for salmonellosis in humans typically ranges from 6 hours to 6 days, accompanied by clinical manifestations such as diarrhea, fever, and abdominal cramps that last for 4 to 7 days (CDC, 2019; FDA, 2019). Severe instances might manifest as high fever, muscle pains, headaches, fatigue, a skin rash, hematuria, or bloody stool, occasionally progressing to a critical stage (FDA, 2019). Diagnosis usually relies on culture-based laboratory assessments of fecal samples, bodily tissues, or fluids. Treatment

primarily involves the administration of broad-spectrum antibiotics, although spontaneous recovery without specific therapeutic intervention is common (CDC, 2019).

### 1.1.2 Microbiological aspects and diagnostic tests

*Salmonella* organisms comprise a cluster of gram-negative prokaryotic bacilli devoid of spore-forming abilities. They possess motile structures known as flagella and fall within the *Enterobacteriaceae* family (Grunberg, 2020; Mumy, 2014). Anchored within a typical *Salmonella* organism are structural components commonly found in bacterial cells, including a capsule, pili, cell wall, plasmalemma, cytoplasm, ribosomes, plasmid, and DNA. Their classification as non-lactose fermenters distinguish them from lactose fermenting counterparts like *Escherichia coli*, *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Serratia* species. Furthermore, *Salmonella* exhibits negative reactions in indole, Voges-Proskauer, and urease tests, while displaying positive reactions in methyl red, citrate, lysine decarboxylase, and ornithine decarboxylase tests (Todar, 2020). It also produces hydrogen sulfide ($H_2S$) upon reaction with thiosulfate (Todar, 2020)

Distinct culture media have been designed for the isolation of *Salmonella* organisms, including Salmonella Shigella (SS) agar, bismuth sulfite agar (BSA), brilliant green agar (BGA), Hektoen enteric (HE) agar, and xylose-lysine-deoxycholate (XLD) agar. This pathogen typically appears as pinkish-white or red colonies on BGA, and black-centered colonies on SS agar, BSA, HE agar, and XLD agar due to $H_2S$ production (Todar, 2020).

Diagnostic methods used in the detection and quantification of *Salmonella* organisms in diverse matrices, ranging from food and water to meat (chicken, pork, beef), feces, cattle hides, and even the lymph nodes (LN) include:

1. Culture-based methodologies: This is known as the gold standard in diagnosis. It involves the use of selective culture media and a range of biochemical tests (as listed earlier) to support the growth and identification of *Salmonella* organisms within a sample (Webb et al., 2017; Wottlin et al., 2022; Nickodem et al., 2023). Culture methodologies offer high specificity and the ability to isolate and quantify pathogens but are time-consuming, labor-intensive, and may have limitations in sensitivity, especially in detecting non-viable microorganisms.
2. Serological tests: Involve the utilization of agglutination and serum antibody assays, both hinging on the fundamental concept of antigen-antibody interactions to ascertain the presence of *Salmonella* (Webb et al., 2017, Atkinson et al., 2019). Serological tests offer rapid results, non-invasive sampling, and the ability to detect past infections. However, they may have limitations in early detection, specificity, and distinguishing between current and past infections.
3. Polymerase chain reaction (PCR): A technique commonly employed to pinpoint specific genes or regions that are unique to *Salmonella*, enabling exceptionally sensitive and specific detection (Bai et al., 2018; Arnold et al., 2023). Depending on the specific type of PCR technique, this method can be used for both qualitative and quantitative analysis. PCR offers high sensitivity, specificity, and rapid results, making it a valuable tool for diagnosing infections, especially in cases where early detection is critical. However, it requires specialized equipment and expertise and may have limitations related to sample inhibitors, contamination, and the inability to distinguish between live and dead pathogens.

4. Enzyme-linked immunosorbent assays (ELISA): This technique serves as another diagnostic test utilized for both quantitative and qualitative analysis. However, it operates by identifying specific antigens (Indirect ELISA) or antibodies (direct ELISA) linked to *Salmonella* within samples (Farzan et al., 2007). ELISA offers high sensitivity and specificity, quantitative results, and rapid turnaround time, making it valuable for diagnosing infections and monitoring immune responses. However, it may have limitations related to cross-reactivity, inability to distinguish current from past infections, and the requirement for specialized equipment and expertise.
5. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS): A rapid and accurate method for the identification of specific bacteria (including *Salmonella*) through the analysis of unique mass spectra of microbial proteins (Yang et al., 2021). MALDI-TOF MS offers rapid and accurate microbial identification capabilities. However, it has initial setup costs and requires specialized expertise.

Less frequently used diagnostic methodologies include next-generation sequencing (NGS), immunoassays, and phage typing.

### 1.1.3 General epidemiology

*Salmonella* currently stands as a significant contributor to the prevalence of foodborne illnesses, particularly in the U.S. (CDC, 2020). Among the significant factors contributing to the overall incidence of human salmonellosis, both within the U.S. and globally is the presence of this pathogen in chicken, pork, and beef. Noteworthy data from the Centers for Disease Control and Prevention reports that about 298 *Salmonella* outbreaks in the U.S. were linked to the consumption of poultry (chicken), yielding 7,881 reported cases of illness, 905 hospitalizations,

and 4 deaths from 1998 to 2017 (CDC, 2020). Of note is the fact that the prevalence and incidence of *Salmonella* in chickens have decreased over time through the implementation of targeted intervention strategies, including vaccination and the enhancement of hygiene practices both at the farm and during processing procedures (CDC, 2020). In contrast, the prevalence of *Salmonella* in ground beef has not exhibited a similar decline. Recent findings from the Division of Foodborne, Waterborne, and Environmental Diseases (DFWED) indicate that since 2017, three distinct outbreaks of *Salmonella* tied to ground beef have been identified. Remarkably, these outbreaks have resulted in as many human illnesses and hospitalizations as the cumulative outbreaks observed over the preceding 36 years. This concerning trend could potentially be attributed to the presence of *Salmonella* serotypes resistant to interventions, and the contamination of ground beef due to dairy cow involvement (CDC, 2020). Previous research has identified the primary cause of ground beef contamination as the presence of *Salmonella* in peripheral LN (Gragg et al., 2013; Webb et al., 2017; Wottlin et al., 2022). As post-harvest interventions seem to hold limited effectiveness in mitigating this issue, intensified efforts have been directed toward the development of robust pre-harvest strategies (Brown et al., 2020; Cernicchiaro et al., 2016; Edrington et al., 2020, Vipham et al., 2015; Wottlin et al., 2022b).

### 1.1.4 Economic impact

The economic impact attributed to *Salmonella* organisms is of major importance, representing another crucial aspect alongside the broader estimation of *Salmonella* prevalence. These factors serve as indicators of the profound consequences associated with human salmonellosis on both a national and global scale. The substantial economic impact of salmonellosis encompasses a range of key aspects, including medical expenses—manifested

through physician office visits, emergency room attendance, outpatient clinic consultations, and hospitalizations—as well as the cost of lost productivity in non-fatal cases and the economic burden of premature death (ERS, 2020).

The U.S. Department of Agriculture's Economic Research Service highlights that the total medical expenses per outcome stood at $38,189,230, $345,911,093, and $2,768,799 for non-hospitalized, hospitalized, and post-hospitalized outcomes, respectively (ERS, 2020). Specifically focusing on hospitalized outcomes, the estimated total medical cost per case reached $17,889 (ERS, 2020). The most substantial contribution to the overall cost of illness attributed to *Salmonella* infections stems from the cost of premature death. The cumulative expenses and per-case cost associated with this aspect were calculated to be $3,667,589,032 and $9,702,616, respectively. It is crucial to recognize that this impact extends beyond human populations, significantly affecting animals as well.

The Cornell University Animal Health Diagnostic Center (2016) underscores the considerable financial strain inflicted by both clinical and subclinical salmonellosis within cattle operations. This impact materializes through a variety of consequences such as decreased milk production, fatalities across all age groups of livestock, abortions, treatment expenditures, increased culling rates, losses from antibiotic-contaminated milk, increased labor requirements for managing sick animals, reduced feed efficiency, and the compromised ability to sell livestock originating from infected herds (Cornell University – Animal Health Diagnostic Center, 2016).

To the beef industry, the economic impact primarily arises from recalls of beef products contaminated with *Salmonella*. Such recalls can result in significant financial losses for meatpacking companies. When contaminated products are identified in the market, the affected batches need to be recalled and removed from store shelves, which can be a costly process. This

cost includes not only the expense of recalling and disposing of the affected products but also the potential loss of sales and damage to a company's reputation. Furthermore, the economic impact extends beyond just the large meatpacking companies. Small businesses within the beef supply chain, such as local ranchers and smaller meat processors, can also suffer when recalls occur. These businesses often operate on thinner profit margins and may not have the financial resources to absorb the costs associated with a recall. A recall could be devastating to a small rancher or a local meat processing facility, potentially leading to financial hardship or even closure.

Appreciating the economic impact brought about by *Salmonella* organisms holds immense value. Such understanding inherently fuels enhanced surveillance methodologies to monitor disease progression. It informs governmental and non-governmental entities in determining priorities for disease management based on the significant impact on both human and animal populations. Moreover, it underscores the importance of supporting research efforts focused on the development of effective intervention strategies to combat this disease (Brichta-Harhay et al., 2020; Mangen et al., 2010).

### 1.1.5 Public health impact – serotype diversity

Among the multitude of *Salmonella* serotypes, unique ones have been singled out for their significant impact on public health, causing severe illnesses within human populations. These serotypes, tied to human salmonellosis, have often been identified in specific host animals (Brichta-Harhay et al., 2020). Nonetheless, it is not uncommon to encounter these serotypes in hosts beyond their natural habitats (CDC, 2014; Webb et al., 2017). An exemplar case is *Salmonella* Enteritidis, frequently isolated from poultry and their byproducts. However, recent

studies have reported the identification of this serotype in other animals like cattle, sheep, goats, and pigs (CDC, 2014). Other serotypes of paramount public health interest encompass *S.* Choleraesuis, most frequently found in pigs, and *S.* Typhimurium, *S.* Newport, and *S.* Dublin, usually found among cattle—particularly cull dairy cattle (Grunberg et al., 2020, Webb et al., 2017). Although these serotypes are of public health significance, they appear at lower frequencies in comparison to other serotypes such as Montevideo, Anatum, Cerro, or Mbandaka. (Webb et al., 2017).

It is noteworthy that *S.* Enteritidis ranks as the most frequently identified serotype during various *Salmonella* outbreaks (Brichta-Harhay et al., 2020). Meanwhile, *S.* Typhimurium stands as the third most common serotype isolated from food sources, contributing to approximately 10% of laboratory-confirmed cases of salmonellosis (CDC, 2018). Notably, certain serotypes like *S.* Typhimurium, *S.* Enteritidis, and *S.* Newport demonstrate the tendency to cause a higher number of human illnesses when compared to other serotypes such as *S.* Dublin and *S.* Choleraesuis (Jones et al., 2008).

The diversity of serotypes is also frequently observed within individual samples collected from animals under study (Cernicchiaro et al., 2016; Webb et al., 2017). Much of the observed serotype diversity in prior studies typically includes at least one of the dominant serotypes within the serotype combination throughout the study duration (Cernicchiaro et al., 2016). However, it remains unclear whether this diversity in individual samples includes serotypes of public health significance (e.g., Newport, Typhimurium), primarily due to their limited occurrence by the end of the study period (Webb et al., 2017).

### 1.1.6 Antimicrobial sensitivity testing and resistance

The contemporary approach within the realm of research revolves around examining the susceptibility of microorganisms to various antimicrobial agents, primarily aimed at addressing the critical issue of antimicrobial resistance, a significant concern for public health. To classify a bacterial isolate as resistant to an antimicrobial agent, it must demonstrate the ability to grow and/or endure in the presence of the specific agent at a concentration exceeding the minimum inhibitory concentration (MIC) using the dilution method (Gragg et al., 2013a; Webb et al., 2017). Alternatively, resistance can be indicated when bacteria closely encircle the antimicrobial agent on the sensitivity plate, forming the zone of inhibition (Britchta-Harhay et al., 2020l; Jorgensen et al., 2015). Previous studies have indicated that resistant isolates are most commonly extracted from clinical salmonellosis cases and have been associated with heightened virulence in comparison to susceptible isolates (Aarestrup, 2006; Britchta-Harhay et al., 2020; Varma et al., 2005).

Addressing the current and potential consequences of antimicrobial-resistant isolates and their impact on public health, the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) was established in 1996. This collaborative effort involves state and local public health departments, the CDC, the U.S. Food and Drug Administration (FDA), and the U.S. Department of Agriculture (USDA). The central objectives of NARMS are to monitor changes in the antimicrobial susceptibility of specific enteric bacteria such as *Salmonella*, *Campylobacter*, *Enterococcus*, and *E. coli* (from chicken, turkey, beef, and pork) derived from sick individuals (CDC), retail meats (FDA), and food animals (USDA) within the U.S. Through its CDC arm, the NARMS program significantly contributes to safeguarding public health by

providing insights into emerging bacterial resistance, the mechanisms driving resistance propagation, and the differences between infections caused by resistant and susceptible strains.

As per the U.S. FDA's 2019 report, while the general level of *Salmonella* resistance remains steady across NARMS samples (from humans, retail meat, and animals at slaughter), notable shifts have occurred in the resistance patterns of certain individual serotypes. For instance, *Salmonella* Infantis, identified as a multidrug-resistant (MDR) serotype back in 2014, emerged as the leading contributor to overall *Salmonella* resistance. This serotype displayed resistance to ceftriaxone and exhibited reduced susceptibility to ciprofloxacin—commonly used in the treatment of *Salmonella* infection (FDA, 2019). Similar trends were evident in *Salmonella* serotype I 4,[5],12:I:-, recognized as a MDR serotype as far back as 2010. However, it is worth noting that only 1% of *Salmonella* isolates from humans showed decreased susceptibility to azithromycin (with most being *Salmonella* Newport), while none of the isolates from animals, animal products, or retail meat demonstrated resistance to colistin (FDA, 2019), suggesting that azithromycin and to a larger extent, colistin, be considered as alternate treatment options for *Salmonella* infections.

Currently, the prevalence of antimicrobial-resistant *Salmonella* isolates in final carcass products remains low due to the effectiveness of existing in-plant intervention strategies (Schmidt et al., 2015).

## 1.2. *Salmonella*: Pre-harvest overview

### 1.2.1 *Salmonella* in cattle

Ruminants, particularly cattle, remain one of the common reservoirs for *Salmonella* organisms (Brichta-Harhay et al., 2020). A systematic review and meta-analysis conducted by

Gutema et al., (2019), revealed a pooled *Salmonella* prevalence of 9% among cattle across different continents within the studies analyzed (Gutema et al., 2019). The highest prevalence of *Salmonella* was documented in North America, with a significant portion of the reports originating from the U.S. (Gutema et al., 2019). This aligns with findings by Heredia et al., (2018), who noted a consistent increase in outbreaks of human salmonellosis linked to beef consumption in the U.S. since the 1980s (Heredia et al., 2018). Within adult cattle, the prevalence of *Salmonella* tends to be notably higher than in calves (Cummings et al., 2010; Gutema et al., 2019). This discrepancy might partially be attributed to the transfer of maternal antibodies from dam to calf, which aids in warding off infections. In essence, the younger the calves, the more resilient they are to infections; however, as they mature, their susceptibility increases (Chase et al., 2008).

Common *Salmonella* serotypes frequently isolated from cattle encompass Montevideo, Typhimurium, Kentucky, Meleagridis, Anatum, Cerro, Mbandaka, Muenster, Newport, and Senftenberg. Interestingly, North America demonstrates the highest frequency of reported *Salmonella* infections characterized by the serotype Montevideo, while it also ranks second globally for the greatest diversity of serotypes (Gutema et al., 2020).

Cattle generally contract *Salmonella* infections through contaminated food and water or, at times, via direct contact with an infected animal. However, salmonellosis in cattle often manifests in a subclinical form, resulting in the absence of noticeable clinical signs. Consequently, diagnosing cattle salmonellosis predominantly relies on the culture and isolation of fecal samples, hides, and, as more recent reports suggest, LN.

### 1.2.2 Prevalence in feces

The gastrointestinal tract, specifically the small intestine, is a recognized predilection site for *Salmonella* organisms which consequently accounts for their consistent presence and subsequent shedding in feces (Grunberg, 2020). Extensive investigations have reported on *Salmonella* prevalence in cattle, with a particular focus on fecal samples (Callaway et al., 2006; Cernicchiaro et al., 2016; Dodd et al., 2011; Lauren et al., 2022; Loneragan et al., 2012).

For instance, in a study conducted by Callaway et al., (2006), a total of 960 fecal samples were collected from lactating dairy cows spanning four states within the U.S. Their investigation revealed a *Salmonella* prevalence of 9.96% (93/960), predominantly concentrated in the Northeastern region of the country (Callaway et al., 2006). Loneragan et al., (2012), extended their investigation to cull dairy cattle in Dalhart, Texas, during the months of June through September. Their analysis of 706 fecal samples revealed a notable prevalence of 32.6% (Loneragan et al., 2012). Cernicchiaro et al., (2016), delved into *Salmonella* prevalence across a feedlot trial setting within the Texas panhandle, gathering a substantial dataset of 1,558 samples. Their findings demonstrated higher mean fecal prevalence of *Salmonella* at 64.2% (1,000/1,558) across diverse sampling months (Cernicchiaro et al., 2016). This increased prevalence was mirrored in a commercial feedlot study in Texas in 2011, wherein the mean fecal prevalence of *Salmonella* stood at 64.7% upon feedlot arrival and increased to 72.6% just prior to harvest, encompassing all cohorts under observation (Dodd et al., 2011).

### 1.2.3 Prevalence in hides

Cattle hides stand out as another favored location for *Salmonella* colonization (Gragg et al., 2013b). The presence of *Salmonella* on cattle hides primarily arises from fecal or

environmental contamination (Madoroba et al., 2016). This, in turn, poses the risk of subsequent carcass contamination during processing, ultimately leading to the contamination of ground beef (Gragg et al., 2013b). Despite various management interventions practiced by different farms, the inherent behaviors of cattle—such as frequent movement, mingling with peers, and exposure to potentially contaminated surroundings—have maintained the prevalence of *Salmonella* on hides at a relatively consistent level (McEachran et al., 2015).

Previous studies have all documented large *Salmonella* prevalence estimates, ranging from 70.8% to 100% (Barkocy-Gallagher et al., 2003; Gragg et al., 2013b; Koohmaraie et al., 2012; Brichta-Harhay et al., 2008 and 2011; Rivera-Bentancourt et al., 2004). These findings underscore the substantial degree of *Salmonella* contamination evident on cattle hides. As suggested by Wheeler et al., (2014), plausible explanations for this increased prevalence could be traced to cross-contamination that often occurs between animals during transportation from farms to abattoirs and within the lairage period.

### 1.2.4 Prevalence in food and water

Food and water serve as the major route through which *Salmonella* organisms are transmitted to other animals within a herd (Grunberg, 2020). The persistent presence of *Salmonella* in cattle's feeding and water troughs can be attributed to several factors. These encompass infected animals consistently utilizing these areas for feeding and water consumption, along with the potential deposition of fecal matter (Grunberg, 2020; LeJeune et al., 2001). Furthermore, non-cattle vectors like rodents, wild birds, and flies contribute to this persistence through defecation in feeding and water troughs (Grunberg, 2020; Carlson et al., 2011). The contamination of major water sources also serves as a pathway, leading to the widespread contamination of water troughs throughout the farm (Brichta-Harhay et al., 2020). Additional

sources include feed contamination resulting from the use of animal waste or *Salmonella*-contaminated water as fertilizers for crops (Dargatz et al., 2005; Troth et al., 2011). Moreover, contamination might occur during storage, transportation, and handling processes (Brichta-Harhay et al., 2020). A comprehensive study on *Salmonella* prevalence in cattle was carried out by Edrington et al., (2008), which encompassed various matrices including fecal samples, soil samples, total mixed ration (TMR) (feed), and water samples (Edrington et al., 2008). While the prevalence of *Salmonella* in water samples demonstrated considerable variation, ranging from 0% to 75% with a mean of 38%, *Salmonella* prevalence in the TMR exhibited an even broader spectrum, ranging from 0% to 100% with a mean prevalence of 76% (Edrington et al., 2008). Notably, instances of increased *Salmonella* prevalence in the TMR did not consistently correlate with that observed in fecal samples (Edrington et al., 2008).

## 1.3 Risk factors associated with *Salmonella* prevalence in cattle

### 1.3.1 Cattle production types

Previous studies have identified cattle production types as a significant risk factor in the prevalence of *Salmonella* within cattle populations (Webb et al., 2017; Wottlin et al., 2022a). It is also apparent that among cattle types, feedlot cattle (primarily raised for meat production) exhibit a higher *Salmonella* prevalence compared to dairy cattle (primarily bred for milk production). This observation is underscored by the findings of Webb et al., (2017), where an extensive study investigating *Salmonella* prevalence was conducted across distinct cattle production sources, various seasons, and diverse regions across the U.S. (Webb et al., 2017). In their study, a prevalence of 7.1% was recorded among feedlot cattle, while cull dairy cattle demonstrated a prevalence of 1.8% (Webb et al., 2017). These findings were paralleled by those reported by Gragg et al., (2013), where *Salmonella* prevalence of 14.7% and 1.8% were

identified among feedlot and cull cattle, respectively (Gragg et al., 2013a). Gragg et al., (2013b) and Levent et al., (2019) also recorded similar outcomes, reporting a 75% *Salmonella* prevalence among feedlot-fattened cattle. A comprehensive meta-analysis and systematic review conducted by Gutema et al., (2019) covering *Salmonella* prevalence and serotype diversity in apparently healthy cattle from 2000 to 2017 yielded corroborative results, indicating that cattle from feedlot operations exhibited the highest *Salmonella* prevalence compared to other production sources (Gutema et al., 2019).

A likely explanation for this observed disparity can be attributed to the age of the animals at the time of slaughter. In the U.S., feedlot cattle are typically slaughtered at two years of age or younger, affording them relatively less time to develop a robust immune system when compared to dairy cattle that are typically culled and slaughtered at an older age (Webb et al., 2017). Additionally, it is plausible that early exposure of dairy cattle to *Salmonella* organisms imparts a stronger immune response, rendering them more resistant compared to animals without prior exposure. However, instances of higher *Salmonella* prevalence in cull dairy animals have also been documented. Notably, Wottlin et al., (2022), reported increased *Salmonella* prevalence in fecal samples from cull dairy cattle (48.8%) in comparison to the other four production systems (cull beef, conventional feedlot, grass-fed, and all-natural feedlot) examined in their study (Wottlin et al., 2022a). The precise reasons for the heightened fecal prevalence among dairy cattle remain unknown but likely arise from increased physiological stress due to lactation and parturition periods (Edrington et al., 2004).

### 1.3.2 Season

A strong association has been established between *Salmonella* prevalence and seasonal variations. *Salmonella* organisms thrive optimally within incubational temperature range of 35°C to 43°C, where their metabolic activities are most productive (ICMSF, 1996). Furthermore, their ability to survive is observed under optimal water activity conditions of 0.99, allowing their persistence even in low-water-activity foods such as chocolate and peanut butter (ICMSF, 1996; Podolak et al., 2010). Drawing from the analysis of *Salmonella* case data from the Maryland Foodborne Diseases Active Surveillance Network (2002-2012) and weather data from the National Climatic Data Center (1960-2012), Jiang et al., (2015) reported a noteworthy connection between salmonellosis risk and meteorological factors. They reported a 4.1% increase in salmonellosis risk for each unit rise in ambient temperature and a 5.6% increase associated with each increment in precipitation events (Jiang et al., 2015). Employing a switching model, Stephen et al., (2016), also reported associating temperature increases of 5°C with a 45.4% rise in salmonellosis cases and 10 mm precipitation increments with a 24.1% increase.

Previous investigations have consistently reported increased *Salmonella* prevalence in feces and hides among cattle during the warmer months compared to other months within a year (Cernicchiaro et al., 2016; Kunze et al., 2008; Levent et al., 2019; Likavec et al., 2016; Wottlin et al., 2022a). This recurring trend aligns with the conducive environment created by higher temperatures during summer and, in certain cases, fall (Kendrovski et al., 2011; Wottlin et al., 2022a). These conditions provide an optimal environment for *Salmonella* organisms to thrive. Nevertheless, Kunze et al., (2008) found no significant seasonal variation in *Salmonella* prevalence among cattle (Kunze et al., 2008). Despite this, the plausible explanation for the

seasonal trend rests on the increased temperatures during these warmer periods, which facilitate the proliferation of *Salmonella* (Kendrovski et al., 2011; Wottlin et al., 2022a).

The influence of non-mammalian vectors, specifically biting flies, in the transmission of salmonellosis, warrants attention (Grunberg et al., 2020). These vectors exhibit a higher prevalence during the warmer seasons, thereby augmenting the potential for increased salmonellosis cases due to their interactions with the host animals (Taylor et al., 2012; Edrington et al., 2013a, b; Xu et al., 2017; Olafson et al., 2016).

### 1.3.3 Region

Geographical variations have consistently played a role in influencing *Salmonella* prevalence among animals, including cattle (Brichta-Harhay et al., 2020). To facilitate effective monitoring, the Beef Industry Food Safety Council has thoughtfully delineated the U.S. into distinct microbiological monitoring regions, each tailored to guide the selection of appropriate samples and the specific animal or production source under study. This approach has proven instrumental in comprehending *Salmonella* prevalence dynamics. For instance, the Western and Midwest regions (BIFSCo regions 2 and 6, respectively) of the U.S. are predominantly characterized by dairy farms. Consequently, one can anticipate increased *Salmonella* prevalence in milk samples, as well as in feces and LN collected from dairy cattle, relative to other production sources. Conversely, in the case of feedlot cattle, which predominantly inhabit the Southwest regions of the U.S. (BIFSCo region 3), distinct prevalence patterns emerge. While Callaway et al., (2006), reported higher fecal prevalence among farms in the Northeastern U.S. (BIFSCo region 8), a majority of previous studies have consistently revealed higher prevalence

in the Southwest regions compared to other areas within the U.S. (Callaway et al., 2006; Edrington et al., 2008; Wells et al., 2001; Wottlin et al., 2022a).

A prominent rationale for these disparities revolves around the characteristic high environmental temperatures typically associated with Southern and Southwestern regions (Brichta-Harhay et al., 2020). Additionally, the increased presence of non-mammalian vectors such as wild birds and flies, facilitated by the region's environmental temperature, could further compound *Salmonella* prevalence variations (Johnson, 2020; Taylor et al., 2012).

### 1.3.4 Cattle class and breeds

Diverse classes and breeds of cattle exhibit varying degrees of susceptibility and resistance to *Salmonella* organisms. The two broad categories of cattle are the *Bos indicus*, commonly distributed across tropical regions such as Africa and Southeast Asia, and the *Bos taurus*, predominantly found in temperate zones like the U.S. Within these broad classifications, numerous distinct breeds share characteristic traits associated with their specific cattle type (Mandalena et al., 2019).

Literature underscores the fact that cattle originating from tropical regions are more resistant to infections compared to cattle in temperate regions (Mandalena et al., 2019). This could also explain the increased prevalence of *Salmonella* organisms documented in North America, particularly the U.S. However, other plausible reasons include the high number of reports and publications consisting of 33,577 cattle samples, and the different monitoring and surveillance mechanisms emanating from the U.S. (Gutema et al., 2019).

In other continents such as Africa and South America, crossbreeding between these two classes of cattle, is a common practice with the aim of reducing the limitations of each cattle

class, while maintaining or increasing their productivity (Gama et al. 2003; Mandalena et al. 2019). However, this may not be the case in the U.S., crossbreeding between different breeds belonging to the same class of cattle (*Bos taurus*) is commonly practiced, with more emphasis on dairy production (Cassell et al. 2009).

### 1.3.5 On-farm management systems

Different management strategies are implemented by cattle farms across various regions within the U.S. These strategies can impact the prevalence and concentration of *Salmonella* within the herd. Brichta-Harhay et al., (2020) and Grunberg, (2020), describes different on-farm practices, ranging from comprehensive herd-wide vaccination initiatives, the selection and administration of antibiotics, and the incorporation of specific feed additives, designed to mitigate *Salmonella* presence. Other relevant biosecurity measures include regularly sanitizing animal housing, feeding and water troughs, frequently removing animal waste to prevent feed and water supply contamination, restricting visitor to individual pens, and prioritizing personal hygiene protocols among farm personnel. Additional measures may involve sourcing cattle from reputable establishments that are verified to be free of salmonellosis. Such cattle would typically be placed on quarantine before being allowed to joined the existing herd. Containment efforts might also entail the culling of infected animals and adequate testing of feed and feedstuffs to ensure they are from *Salmonella* contamination (Grunberg, 2020).

Certain cattle farms may also practice a multi-hurdle approach, combining several management strategies to prevent disease occurrence in the herd (Wheeler et al., 2014). interestingly, those farms that possess a streamlined and efficient management system invariably

exhibit reductions in both the prevalence and concentration of *Salmonella* in comparison to their counterparts with less comprehensive protocols (Loneragan et al., 2012; Wilhelm et al., 2017).

## 1.4 Pre-harvest intervention strategies for *Salmonella*

### 1.4.1 Vaccination

The use of vaccines stands as a prominent defense strategy in preventing infections within a cattle herd. Vaccination in animals involves the administration of antigens, which are nonpathogenic yet immunogenic components, designed to stimulate or enhance the immune response of a host animal. The primary reason for vaccination is to confer protection against subsequent infections in the near future (Brichta-Harhay et al., 2020; Grunberg, 2020). Vaccines can be produced in live or inactivated forms based on the manufacturer and the nature of the targeted infection (Grunberg, 2020). Given the intracellular nature of *Salmonella* infections, live vaccines are deemed essential for inducing robust immunity. Nevertheless, successful experimental applications of inactivated vaccines against *Salmonella* have also been demonstrated (Grunberg, 2020). Notably, commonly employed *Salmonella* vaccines encompass the *Salmonella* Newport bacterial extract SRP, *Salmonella* Dublin vaccine, *Salmonella* Typhimurium bacterin-toxoid vaccine, and combinations thereof, such as the *Salmonella* Dublin and *Salmonella* Typhimurium Vaccine (USDA-APHIS, 2022).

In the U.S., while vaccination is extensively employed and effective against various diseases in cattle, it is less effective in mitigating *Salmonella* infections. Data from the U.S. Department of Agriculture – Animal and Plant Health Inspection Service in 2013, reveals that fewer than 7% of feedlots utilize *Salmonella* vaccines, and merely about 10% of dairy farms incorporate *Salmonella* vaccination based on the latest study conducted in 2007 (USDA-APHIS,

2009 & 2013a). Previous studies, including a rapid systematic review and meta-analysis, have demonstrated inconclusive or non-significant positive outcomes pertaining to the impact of vaccination against *Salmonella* in cattle (Cernicchiaro et al., 2016; Dodd et al., 2011; Wilhelm et al., 2017). These findings underscore key reasons behind the limited adoption of *Salmonella* vaccines among feedlot and dairy farms. However, Loneragan et al., (2012) demonstrated *Salmonella* prevalence reductions in vaccinated animals compared to their counterparts. Additionally, several authors reported increased levels of *Salmonella* antibodies in milk and higher seroconversion rates through the utilization of *Salmonella* Typhimurium mutant bacterin-toxoid, *Salmonella* Newport, and *Salmonella* Dublin vaccines, respectively (Miller et al., 1991; Smith et al., 2014; Smith et al., 2015).

### 1.4.2 Use of direct-fed microbials

According to the Office of Regulatory Affairs of the Food and Drug Administration (FDA) and the Association of American Feed Control Officials, direct-fed microbials (DFM) are considered products containing live and/or viable micro-organisms typically introduced into animal diets to curtail the population of foodborne pathogenic agents (Brashears et al., 2005). The application of DFM stands as a straightforward and convenient intervention strategy, involving their incorporation into the animal's feed alongside other dietary constituents. This approach can be combined with other pre-harvest intervention tactics, such as biosecurity measures, for enhanced efficacy (Brichta-Harhay et al., 2020). Distinct categories of DFM exist, each exerting specific modes of action, largely indirect, in countering pathogens within host animals (McAlister et al., 2011). Commonly employed DFM encompass bacterial species like *Lactobacillus acidophilus*, *Bacillus subtilis*, *Bifidobacterium bifidum*, *Propionibacterium*

*freudenreichii*, and yeast/fungi such as *Saccharomyces cerevisiae* and *Aspergillus oryzae* (McAlister et al., 2011; Quigley et al., 2011). The précised mechanism of action of these organisms are still not well understood, however, researchers have defined different school of thoughts such as competition with harmful bacteria in the GIT for nutrients thereby decreasing the survival rate of harmful pathogens in the gut, production of organic acids within the GIT which will lead to a lowered gut pH and consequently pathogen inhibition, production of hydrogen peroxide which inhibits the growth of harmful bacteria through its cytotoxic effect on bacterial cells, competition for adhesion sites on the intestinal epithelium which consequently prevents colonization of pathogens, and/or stimulation of both mucosal and systemic immune responses, enhancing the phagocytic activities of immune cells against pathogenic bacteria like *Salmonella* (Brashears et al., 2005; Feye et al., 2016 & 2019; Jin et al., 1996, Juven and Pierson, 1996; Nelson et al., 2020, Spencer and Chesson, 1994)

The majority of the previous studies have revealed no substantial association between DFM and reductions in fecal *Salmonella* levels (Smith et al., 2021; Stephens et al., 2007; Tabe et al., 2008). However, Brewer et al., (2014), documented noteworthy declines in *Salmonella* load and virulence within pre-weaned dairy calves upon administering *S. cerevisiae*. Likewise, Feye et al., (2016), reported diminished colonization of *Salmonella* within LN and reduced fecal shedding upon administration of DFM.

### 1.4.3 Bacteriophage (phage) therapy

Bacteriophage-based intervention entails the utilization of bacteriophages, also defined as "virus-eating-bacteria," to mitigate the population of pathogenic bacteria within host animals (Iftikhar, 2019). This strategy hinges on the bacteriophages' unique mode of action, where their

genetic material (DNA or RNA) infiltrates bacterial cells, triggering a prolific replication process yielding over a thousand copies of the phage's genetic material. Subsequently, the bacterial cell succumbs to lysis, releasing newly generated phages into the environment (Iftikhar, 2019).

Xie et al., (2016) documented the potential impact of bacteriophages in reducing *Salmonella* prevalence within the feedlot environment. Interestingly, bacteriophages were present in feedlots lacking *Salmonella* organisms (Xie et al., 2016). This study underscores the potential of bacteriophage intervention as a tool against pathogenic bacteria, paving the way for further research in this intervention approach within livestock environments.

## 1.5 *Salmonella* in peripheral lymph nodes of cattle

There has been a growing recognition of bovine LN as a potential source of ground beef contamination (Arthur et al., 2008a; Gragg et al., 2013a b; Haneklaus et al., 2012; Koohmaraie et al., 2012; Webb et al., 2017; Wottlin et al., 2022a). This insight emerged due to the presence of *Salmonella* organisms in ground beef, even in the face of effective post-harvest strategies employed by various commercial processing facilities (Arthur et al., 2008a; Haneklaus et al., 2012; Webb et al., 2017). Consequently, recent research works have shifted their focus toward examining the prevalence of *Salmonella* in bovine LN, with particular attention on the PLN. The exploration of the mechanisms governing the entry, survival, and replication of *Salmonella* within these LN is essential in uncovering potential ways for reducing *Salmonella* contamination in beef products (Brichta-Harhay et al., 2020).

### 1.5.1 Bovine lymph nodes

Lymph nodes serve as vital protective reservoirs within the body, primarily situated within adipose tissues. These nodes house an array of immune cells and operate as a filtration organ designed to isolate bacteria, viruses, and other pathogens, which are then targeted for elimination by immune cells like lymphocytes and macrophages (Buettner et al., 2012). Nevertheless, certain bacterial organisms, including *Salmonella*, exhibit a remarkable capacity to evade this immune process. *Salmonella* tends to evade destruction and instead thrives within macrophages, leading to the impairment of immune cells and the overall onset of disease. Given the protective environment provided by LN, the eradication of *Salmonella* organisms during harvest presents a significant challenge, thus explaining the limited effectiveness of post-harvest intervention strategies (Brichta-Harhay et al., 2020).

Prior investigations have predominantly focused on assessing *Salmonella* prevalence within the mesenteric LN. These studies have extensively examined the entry pathways, prevalence, concentrations, and associated risk factors for *Salmonella* survival within LN situated within the gastrointestinal tract (Samuel et al., 1980 and 1981; Sofos et al., 1999). However, with *Salmonella* prevalence in ground beef products remaining at a relatively constant level, and considering that mesenteric LN are typically discarded during the evisceration process, recent research works have shifted their attention to PLN. These LN, situated at the periphery, hold a higher likelihood of being included in beef products, making them a pertinent focal point for investigations (Gragg et al., 2013a b; Webb et al., 2017; Wottlin et al., 2022a).

### 1.5.2 Route of entry

The precise route through which *Salmonella* enters the PLN remains a subject of ongoing investigation, though various studies have proposed potential avenues including the gastrointestinal system, transdermal route, and transmission through disease vectors such as biting flies (Edrington et al., 2013a, b, c; Gragg et al., 2013b; Olafson et al., 2014).

The lymphatic system, a complex network, features several distinct branches that facilitate the drainage of lymph into LN from distinct regions of the body (Brichta-Harhay et al., 2020). Different types of PLN receive lymph from specific areas; the subiliac LN filter lymph from the skin of the abdominal wall, hind limbs, and pelvis, the superficial cervical/pre-scapular LN filter lymph from the forelimbs, cervical area, and parts of the thoracic region, and the popliteal LN undertake lymph filtration from the hind limbs (Figure 1) (Edrington et al., 2013a, b; Gragg et al., 2013b; Porwollik et al., 2018). *Salmonella* organisms are commonly harbored within *Salmonella* founder cells, primarily residing in the gastrointestinal tract (Porwollik et al., 2018). While *Salmonella* isolates often originating from the gastrointestinal tract can be detected in PLN, a study by Porwollik et al., (2018) revealed a limited presence of *Salmonella* founder cells within these nodes. Investigating the potential of the gastrointestinal route for transmission, an experimental study by Edrington et al., (2013a), described the impact of oral administration of *Salmonella* and their subsequent presence in PLN. Their findings indicated that although *Salmonella* administered orally could be found in PLN, the concentrations used in the experimental setup might not represent those encountered in natural conditions, accounting for the relatively low concentrations of *Salmonella* organisms observed in PLN.

In contrast to the oral challenge, Edrington et al., (2013a) examined the transdermal route as an alternative mode of *Salmonella* transmission to PLN. They reported *Salmonella* recovery in

58.3% of all the PLN and in at least one of the PLN from 93.8% of the calves (Edrington et al., 2013a). The transdermal route demonstrated a regionally specific and predictable *Salmonella* recovery from PLN when compared to the oral route. Further refining this approach, Edrington et al., (2013b) developed a transdermal *Salmonella* challenge model in calves, yielding consistent *Salmonella*-positive PLN. Their research revealed successful recovery of *Salmonella* from region-specific PLN for up to 8 days post-challenge, with specific serotypes recovered from PLN corresponding to the inoculated regions (Edrington et al., 2013b).

An additional mode of transmission involves vectors associated with foodborne illnesses, such as biting flies, known to harbor *Salmonella* organisms in their mouthparts and digestive tracts (Olafson et al., 2014). These vectors contribute to *Salmonella* transmission in two ways: firstly, by carrying *Salmonella* on their mouthparts during their interactions with the animal's body, and secondly, by causing injuries and abrasions on the skin that expose the animal to various microorganisms including *Salmonella* found on hides (Olafson et al., 2014; Webb et al., 2017). Olafson et al., (2014) identified horned flies (*Haematobia irritans*) as an example of biting flies capable of penetrating the skin and potentially facilitating the transmission of *Salmonella* to regional PLN.

### 1.5.3 Epidemiological trends of *Salmonella* in peripheral lymph node

The evaluation of *Salmonella* prevalence within specific LN predominantly focuses on the subiliac, superficial cervical, and popliteal LN due to their larger size, ease of harvest, higher likelihood of containing *Salmonella* organisms, and potential incorporation into ground beef (Brichta-Harhay et al., 2020; Webb et al., 2017).

Previous studies have investigated the prevalence, concentration, and associated risk factors of *Salmonella* within the PLN of cattle. Gragg et al., (2013a) conducted a cross-sectional study on *Salmonella* carriage in subiliac iliac LN of cull and feedlot cattle at harvest, reporting an overall *Salmonella* mean prevalence of 7.5% among 3,327 samples. They observed higher prevalence in feedlot cattle compared to cull cattle, and a seasonal variation, being higher during warmer seasons and in the Southwestern regions (Gragg et al., 2013a). Similar trends were reported by Webb et al., (2017), and Wottlin et al., (2022a), with *Salmonella* prevalence influenced by season, region, and animal type. This mirrors earlier findings in cattle feces and hides (Cernicchiaro et al., 2016; Edrington et al., 2004; Wottlin et al., 2022a). Gragg et al., (2013b) also extended their research to consider other LN within the animal's body in addition to subiliac and mesenteric LN. They reported that while *Salmonella* was isolated from 91.2% and 76.5% of mesenteric and subiliac LN samples, respectively, a recovery rate of 55.9% and 7.4% was recorded in mandibular and mediastinal LN samples respectively (Gragg et al., 2013b)

Examining *Salmonella* concentrations within individual nodes has also gained interest, often with a quantification limit set at 1-1.6 $\log_{10}$ CFU/PLN (Edrington et al., 2020; Gragg et al., 2013a; Webb et al., 2017; Wottlin et al., 2022a). Gragg et al., (2013a) observed 23.3% of cattle with pre-enriched *Salmonella*, particularly during warmer seasons, with 18.4% exceeding the quantification limit (~1.0 $\log_{10}$ CFU/PLN). Webb et al., (2017) reported *Salmonella* concentrations ranging from 1.6-4.9 $\log_{10}$ CFU/PLN, and 17.6% of positive PLNs harboring *Salmonella* >3.0 $\log_{10}$ CFU/PLN, mostly from feedlot cattle during warmer seasons, a similar finding reported by Gragg et al., (2013a). A recent study by Wottlin et al., (2022a) on *Salmonella* carriage in PLN and feces revealed concentrations within individual LN (0.1-3.8 $\log_{10}$ CFU/g of LN) influenced by cattle type, region, and season. Higher *Salmonella* concentrations were

identified in conventional feedlot cattle, particularly during warmer seasons and regions within the U.S.

The presence of *Salmonella* serotypes and their antimicrobial resistance within cattle PLN has also been explored (Cernicchiaro et al., 2016; Gragg et al., 2013a b; Webb et al., 2017). Previous investigations have identified a range of distinct *Salmonella* serotypes (between 22 to 24), with dominant serotypes including Montevideo, Anatum, Lille, and Cerro (Gragg et al., 2013b; Webb et al., 2017). While serotypes of public health significance, such as *S.* Typhimurium, *S.* Newport, and *S.* Dublin, are often in the minority and present at low concentrations, their potential impact on human health is notable when compared to other serotypes (Webb et al., 2017).

The issue of antimicrobial resistance is increasingly critical, given the increasing resistance levels to various antimicrobial agents. As elucidated by Gragg et al., (2013), and Webb et al., (2017), the majority of *Salmonella* isolates remained pan-susceptible, with percentages ranging from 80% to 86% (Gragg et al., 2013b; Webb et al., 2017). However, an upward trend in the prevalence of MDR isolates has been documented, rising from 8.3% to 10.7% between 2013 and 2017 (Gragg et al., 2013b; Webb et al., 2017). This shift is predominantly observed among isolates from cull animals in contrast to those from feedlot animals. This could be due to the increased antimicrobial therapy being administered to the animals prior to culling or due to the increased age of the animals presenting the opportunity of longer antimicrobial therapy administration eventually leading to increased MDR isolates (Wottlin et al., 2022).

### 1.5.4 Potential intervention strategies

In recent years, considerable focus has been directed towards establishing efficient intervention strategies to mitigate *Salmonella* organisms in the PLN of cattle, thereby preventing their transmission to humans through the consumption of ground beef. One of the initial propositions involved the complete removal of all LN during the harvesting process (Brichta-Harhay et al., 2020). However, given the multitude of distinct PLN in animals, this approach was modified to target the extraction of larger and more accessible PLN during fabrication (Brichta-Harhay et al., 2020). This modification prompted the Federal Purchase Ground Beef Program, under the directives of the USDA Agricultural Marketing Services, to implement protocols focusing on the removal of prominent PLN (such as subiliac, pre-scapular, and popliteal) in an endeavor to diminish *Salmonella* prevalence within commercial processing plants, primarily for the National School Lunch Program (USDA-AMS, 2013).

Yet, beyond the presence and survival of *Salmonella* within PLN, these LN confer a degree of protection against several intervention strategies employed at processing plants. Whereas methods like irradiation might reduce prevalence within LN, the lack of wide consumer acceptance, coupled with limited efficacy of other post-harvest interventions, has steered recent efforts towards generating effective pre-harvest measures that can be applied on farms to reduce *Salmonella* prevalence in PLN, consequently safeguarding ground beef quality (Cernicchiaro et al., 2016; Edrington et al., 2013 and 2020; Vipham et al., 2015; Wottlin et al., 2022b). Various pre-harvest interventions encompassing vaccination, DFM, antimicrobial employment, and bacteriophage therapy have all undergone experimental trials on cattle in both feedlot and dairy settings.

Regarding vaccination, an initial study by Edrington et al., (2013), on the establishment of challenge models to evaluate the efficacy of vaccinations in reducing *Salmonella* prevalence within PLN reported a modest vaccine effect, particularly in specific LN under certain experimental conditions. In 2016, a subsequent experimental study yielded no discernible distinction between vaccinated and control animals, suggesting potential factors such as overwhelming *Salmonella* exposure, cross-protection limitations across serotypes, and inadequate immunity duration relative to harvest (Cernicchiaro et al., 2016). More recent data from Edrington et al., (2020), showed similar findings from the initial study in 2013, as it demonstrated partial vaccine effects in particular scenarios, evaluating the efficacy of two commercially available vaccines in reducing *Salmonella* in PLN.

DFM have also established their effectiveness in reducing disease prevalence across diverse production settings. Vipham et al., (2015), corroborated the impact of DFM against *Salmonella*, highlighting that the supplementation of *Lactobacillus animalis* (NP51) and *Propionibacterium freudenreichii* (NP24) at $10^9$ CFU/head/day correlated with decreased *Salmonella* prevalence and concentration in subiliac LN. Similarly, Feye et al., (2019), observed significant reductions in *Salmonella* LN infiltration, invasiveness, and antibiotic resistance in both cattle and poultry upon administering *Saccharomyces cerevisiae* fermentation product.

Whereas the use of bacteriophages is an intervention strategy still in its developmental stages, it remains a prospective strategy against *Salmonella* within PLN. In contrast, the frequent application of antimicrobials is now strongly discouraged due to the increasing resistance exhibited by *Salmonella* organisms.

## 1.6 *Salmonella*: Post-harvest overview

While understanding that the pre-harvest overview is important, an equally indispensable aspect lies in the post-harvest overview concerning *Salmonella*, encompassing its prevalence, concentration, and the intervention strategies established against its presence in ground beef before it reaches final consumption. *Salmonella* prevalence in beef and dairy carcasses and its eventual presence in ground beef has been a growing concern despite the implementation of several pre-harvest interventions (Brichta-Harhay et al. 2020). With cattle hides being a major predilection site for *Salmonella* organisms, contamination of carcass surface through the process of hide removal is commonly observed in processing plants (Brichta-Harhay et al. 2008). Cross-contamination is also very likely to occur during the fabrication process (Brichta-Harhay et al. 2020). In light of these potential sources of contamination, it becomes apparent that, in the absence of effective post-harvest intervention strategies, a substantial number of human salmonellosis cases may ensue (Brichta-Harhay et al., 2020).

### 1.6.1 Prevalence in beef carcasses

It is widely recognized that a significant proportion of ground beef contamination stems from direct contact with the surface of beef carcasses or the carcasses themselves. This contamination can potentially be traced back to animal feces and/or the environment, which subsequently contaminate the hides, thus initiating surface contamination on the carcasses.

A study conducted by Barkocy-Gallagher et al., (2003), pertaining to seasonal *Salmonella* prevalence across three Midwestern fed-beef processing plants, revealed an overall *Salmonella* prevalence of 4.4%, 71.0%, and 12.7% in feces, hides, and pre-evisceration samples, respectively (Barkocy-Gallagher et al., 2003). This underscored the importance of hides as a major source of

*Salmonella* on beef carcasses. They also reported that after post-harvest intervention strategies were implemented, *Salmonella* was recovered from only one carcass out of the 1,016 sampled (Barkocy-Gallagher et al., 2003).

Similarly, Rivera-Betancourt et al., (2004), investigated the prevalence of pathogenic organisms, including *Salmonella*, across hides, carcasses, and facility environmental samples in two sizable beef processing plants spanning the Southern and Northern regions of the U.S. over a five-month period. They reported a higher *Salmonella* prevalence in hide samples, particularly from Southern-region cattle (91.8%), in contrast to Northern-region counterparts (50.3%). Likewise, the prevalence was more pronounced in fence panels of holding pens from the Southern plant compared to the Northern one. Notably, the prevalence of *Salmonella* on carcasses was markedly lower in both Southern (23.3%) and Northern (26.8%) plants. Their findings support strongly the role of post-harvest interventions, as evidenced by a mere 0% and 0.8% *Salmonella* prevalence on carcasses from the Southern and Northern plants, respectively.

Bacon et al., (2002) also conducted an observational study involving eight commercial processing plants, focusing on *Salmonella* prevalence on beef animal hides and carcasses. The overall prevalence stood at 15.4%, which decreased to 1.3% after hide removal and the application of carcass decontamination treatments (Bacon et al., 2002).

More recently, Villarreal-Silva et al., (2016) used surrogate microorganisms of *E. coli* O157:H7 and *Salmonella*, to simulate pathogen cross-contamination at three different processing plants. At each stage of the harvest process and following the implementation of intervention strategies, samples were taken. Samples were also collected from the structural facilities and equipment, while non-inoculated carcasses were sampled and compared with each hide-inoculated carcass for cross-contamination (Villarreal-Silva et al., 2016). According to their

findings, cross-contamination was observed between the non-inoculated and inoculated carcasses along with the transfer of microorganisms from hides and/or carcass surfaces to the environment (Villarreal-Silva et al., 2016). However, following intervention implementation, no microbial surrogates were detected in any processing plants. (Villarreal-Silva et al., 2016).

### 1.6.2 Prevalence in ground beef

In spite of the comprehensive application of post-harvest interventions within processing facilities, the occurrence of *Salmonella* contamination in ground beef remains a persistent challenge, contributing to numerous cases of human illnesses (Brichta-Harhay et al., 2020). As reported by Laufer et al., (2015), a substantial number of outbreaks—1,965 in total—linked to foodborne transmission occurred between 1973 and 2011, with beef accounting for 96 outbreaks resulting in 3,684 cases of illness. Ground beef, specifically, has been identified as a means for the dissemination of foodborne illnesses, such as salmonellosis. Indeed, previous studies have described that 45% (17 out of 38 cases) of beef-related outbreaks from 2002 to 2011 involved ground beef as the implicated source (Laufer et al., 2015). The traditional notion that ground beef contamination originated primarily from carcass surface contamination and subsequent cross-contamination within processing facilities (Bell, 1997; Huffman, 2002) has recently been revised, as studies identify the presence of *Salmonella* within specific cattle LN, notably the PLN, often integrated into ground beef production (Gragg et al., 2013a; Li et al., 2015; Webb et al., 2017).

To estimate *Salmonella* prevalence in ground beef, Bosilevac et al., (2009) examined commercially produced ground beef samples across seven U.S. regions over a 24-month span, reporting an overall prevalence of 4.2%, with approximately 94.2% of samples harboring

concentrations below 2 CFU/g. Correspondingly, Samadpour et al., (2005), and Zhao et al., (2002), documented prevalence estimates of 3.8% and 3.5%, respectively, out of the respective 1,750 and 404 ground beef samples tested.

Furthermore, the identification of pertinent serotypes of public health significance within ground beef samples emerges as a pivotal factor influencing and potentially exacerbating human salmonellosis cases. Zhao et al., (2002), identified eight distinct *Salmonella* serotypes, with *S.* Typhimurium (5) exhibiting the highest prevalence among the 14 isolated strains. Similarly, Bosilevac et al., (2009), highlighted that MDR serotypes, such as *S.* Dublin and *S.* Typhimurium, were among the most prevalent strains during their study. This underscores the increasing concern regarding antimicrobial resistance, necessitating more robust measures for tackling the prevalence of *Salmonella* in ground beef (Zhao et al., 2002; Laufer et al., 2015).

## 1.7 Post-harvest intervention strategies

A combination of post-harvest intervention strategies has been identified from previous studies to be of great efficacy in reducing the prevalence and/or concentration of foodborne pathogens including *Salmonella* organisms in beef destined for human consumption. Among these strategies are:

### 1.7.1 Use of physical strategies

The decontamination of beef carcasses through physical means encompasses a range of techniques including knife trimming, washing, and steam vacuuming (Prasai et al., 1995; Wheeler et al., 2014). While it may be considered less effective when used alone, its efficacy is notably enhanced when integrated with other post-harvest interventions (Brichta-harhay et al., 2020).

Prasai et al., (1995), conducted a study to assess the effectiveness of knife trimming and washing in diminishing microbial populations on beef carcass sides sourced from commercial processing facilities. Their findings indicated that the mean aerobic plate counts (APC) exhibited the most substantial reductions in trimmed samples (3.0 $\log_{10}$ CFU/$cm^2$), followed by trimmed and washed samples (0.9 $\log_{10}$ CFU/$cm^2$), and washed samples (0.3 $\log_{10}$ CFU/$cm^2$), successively, in comparison to untrimmed and unwashed samples (Prasai et al., 1995). This highlights the potential of trimming as a valuable tool for reducing microbial loads in beef. However, the efficacy of washing was found to be relatively limited in reducing APC, possibly attributed to the use of water at lower temperatures and the tendency for washing to disseminate microorganisms to other regions of the beef samples (Prasai et al., 1995).

In a comprehensive analysis of pre- and post-harvest interventions within the U.S. beef industry, Wheeler et al., (2014) highlighted steam vacuuming, among other physical intervention approaches, as an essential means of removing visible contaminants from carcass surfaces at different processing stages for beef.

### 1.7.2 Use of acid antimicrobials

Acidic antimicrobials are frequently integrated into the protocols of most commercial processing facilities due to their capacity for reducing both visible and concealed contamination on beef carcasses (Brichta-Harhay et al., 2020). These antimicrobials encompass compounds such as acetic acid, citric acid, and lactic acid (Wheeler et al., 2014). The exact mechanisms underpinning the antimicrobial effects of organic acids remain an area of ongoing investigation, but they are linked to their ability to disrupt the transmembrane protein gradient and other

cellular structures within microbial cells, leading to cellular injury, diminished activity, and impediment of growth (Wheeler et al., 2014).

Previous literature reveals that within commercial plants, the application of organic acid solutions for washing beef and lamb carcasses before the chilling stage often involves concentrations of 1.5 to 2.5%, although some facilities utilize concentrations as high as 5% (USDA-FSIS, 1996; Wheeler et al., 2014). Enhanced efficacy of organic acids has also been observed when employed for carcass washing/rinsing at temperatures ranging from 50-55$^0$C (Barkate et al., 1993). However, it is important to note that the effectiveness of organic acids can also be influenced by factors like the duration of bacterial contact with meat surfaces and the protection offered by their presence in small cuts or fats (Wheeler et al., 2014).

Among the frequently used organic acids, lactic acid emerges as the most common choice in commercial processing plants due to its favorable cost-effectiveness and contamination-reducing properties (Wheeler et al., 2014). Various studies have demonstrated that a 2% lactic acid solution exhibits varying degrees of bacterial contamination reduction (Ransom et al., 2003; Schmidt et al., 2014). Moreover, better results have been observed when lactic acid is combined with other organic acids (Kalchayanand et al., 2008; Laury et al. 2009). In the context of *Salmonella* contamination, Arthur et al., (2008) reported a reduction of 1.80 $\log_{10}$ CFU/cm$^2$ when lactic acid was employed as a decontaminant (Arthur et al., 2008b). Similarly, Laury et al., (2009) noted a reduction of 1.1 $\log_{10}$ CFU/cm$^2$ of *Salmonella* when a combination of citric and lactic acid was sprayed onto fresh beef samples that were inoculated (Laury et al., 2009). Yang et al., (2017) conducted a study exploring the effect of lactic acid temperature, revealing a significant *Salmonella* reduction of 0.3 $\log_{10}$ CFU/cm$^2$ across a range of temperatures.

### 1.7.3 Oxidizer antimicrobials

Oxidizing antimicrobials are frequently employed in various commercial processing facilities, with notable examples including peroxyacetic acid (peracetic acid), electrolyzed oxidized (EO) water, acidified sodium chlorite (ASC), and hypobromous acid (Wheeler et al., 2014). These antimicrobial agents are proposed to have a multitude of targets within microbial cells and biomolecules, encompassing processes such as peroxidation, cell membrane disruption, oxidation of oxygen scavengers and thiol groups, enzyme inhibition, nucleoside oxidation, impaired energy production, disruption of protein synthesis, and ultimately culminating in cell death (Wheeler et al., 2014).

Of these oxidizing antimicrobials, peracetic acid has demonstrated significant efficacy in reducing concentrations of both *E. coli* (Kalchayanand et al., 2012; Penny et al., 2007; Ransom et al., 2003) and *Salmonella* (King et al., 2005) on carcass surfaces. In recent years, EO water has also emerged as an effective disinfectant for reducing contamination on meat surfaces (Wheeler et al., 2014). Arthur et al., (2008) reported a reduction of 0.57 to 0.75 $\log_{10}$ CFU/$cm^2$ in *Salmonella* concentration when EO water was applied to beef carcasses (Arthur et al., 2008b). Other oxidizing antimicrobial agents, such as ASC, hypobromous acid, and ozone, at specific concentrations, have also been shown to reduce *Salmonella* load on meat carcasses or carcass surfaces (Wheeler et al., 2014).

### 1.7.4 Use of thermal interventions

Heat-based intervention strategies entail the use of increased temperatures to mitigate or eliminate surface contamination on carcasses or meat samples. Among these strategies, steam vacuuming stands out as a prominent technique, characterized by the synergistic application of

both physical (vacuuming) and thermal (hot water) methods (Wheeler et al., 2014). The mechanism underlying the efficacy of hot water involves the deactivation of enzymes alongside the disruption of DNA and degradation of RNA within bacterial cells, including *Salmonella* (Ray, 2001). Demonstrating the effectiveness of this approach, Arthur et al., (2008b) and Huffman, (2002) noted substantial reductions of up to 2.10 $\log_{10}$ CFU/cm$^2$ and 3 $\log_{10}$ cycles, respectively, in the concentration of contaminants, including *Salmonella*, on beef carcasses when subjected to hot water treatment at temperatures of 74°C and 95°C.

Steam pasteurization represents another form of thermal intervention, which is somewhat different from the traditional hot water methods. Notably, steam offers accelerated heating of carcass surfaces due to its increased heat capacity, distinguishing it from hot water at similar temperatures (Wheeler et al., 2014). Previous studies have reported that the application of steam has yielded remarkable results, reducing *Salmonella* concentrations by 3 $\log_{10}$ CFU/cm$^2$ within a mere 10 seconds post-application (Wheeler et al., 2014).

### 1.7.5 Use of non-thermal interventions

Non-thermal interventions, while not as universally embraced as thermal interventions, offer distinct advantages, particularly in maintaining the meat's structural and compositional integrity while concurrently reducing bacterial loads. This is in contrast to thermal treatments, which may risk compromising meat quality due to inherent physical and chemical changes during the process (Brichta-Harhay et al., 2020). Some of the non-thermal interventions currently in use include Electron Beam (E-beam) irradiation, Ultraviolet (UV) light irradiation, Cold Atmospheric Plasma (CAP), and High-Pressure Processing (HPP) (Wheeler et al., 2014).

E-beam and UV irradiation have received considerable attention within poultry production, with studies showing their efficacy in reducing *Salmonella* prevalence in poultry products. For instance, Lewis et al., (2002) conducted a study investigating the impact of E-beam irradiation at specific doses on skinless chicken breast. Their findings indicated that all chicken breast samples exposed to irradiation demonstrated the absence of *Salmonella* organisms (Lewis et al., 2002). Similarly, UV irradiation also demonstrated its effectiveness as it was observed to reduce *S*. Typhimurium on chicken breast samples by 1.19 log10 CFU/cm2 (Chun et al., 2010).

### 1.7.6 A combination of interventions

It is important to recognize that no single intervention strategy can be deemed universally effective in mitigating bacterial organisms, particularly *Salmonella*. Reasons for this have been attributed to variations in bacterial susceptibility to interventions and the uneven nature of beef carcass surfaces, which can hinder direct contact between the bacterial organisms and the intervention agents (Brichta-Harhay et al., 2020). Consequently, current emphasis is placed on adopting a multi-hurdle approach, wherein diverse intervention strategies are sequentially applied. This comprehensive approach is anticipated to yield more substantial outcomes in reducing contamination levels on meat samples or carcass surfaces (Wheeler et al., 2014; Yeh et al., 2017).

## 1.8 Conclusion

*Salmonella enterica* stands as an important pathogenic bacterium of significant public health concern, given its widespread prevalence. Its presence has been notably identified not only within the feces and hides of cattle but also, of recent paramount importance, within the

PLN of these animals. The intricate interplay of various risk factors (season, region, cattle production sources, cattle type, on-farm management factors) distinctly contributes to the prevalence and concentration of *Salmonella* across these diverse matrices. Whereas previous investigations have been conducted with the aim of formulating both pre-harvest and post-harvest intervention strategies to curtail its prevalence and concentration, a more comprehensive understanding of the route of entry, mechanisms of infection, and the persistent survival of *Salmonella*, particularly within the confines of PLN, holds the potential to yield more effective approaches for pathogen mitigation.

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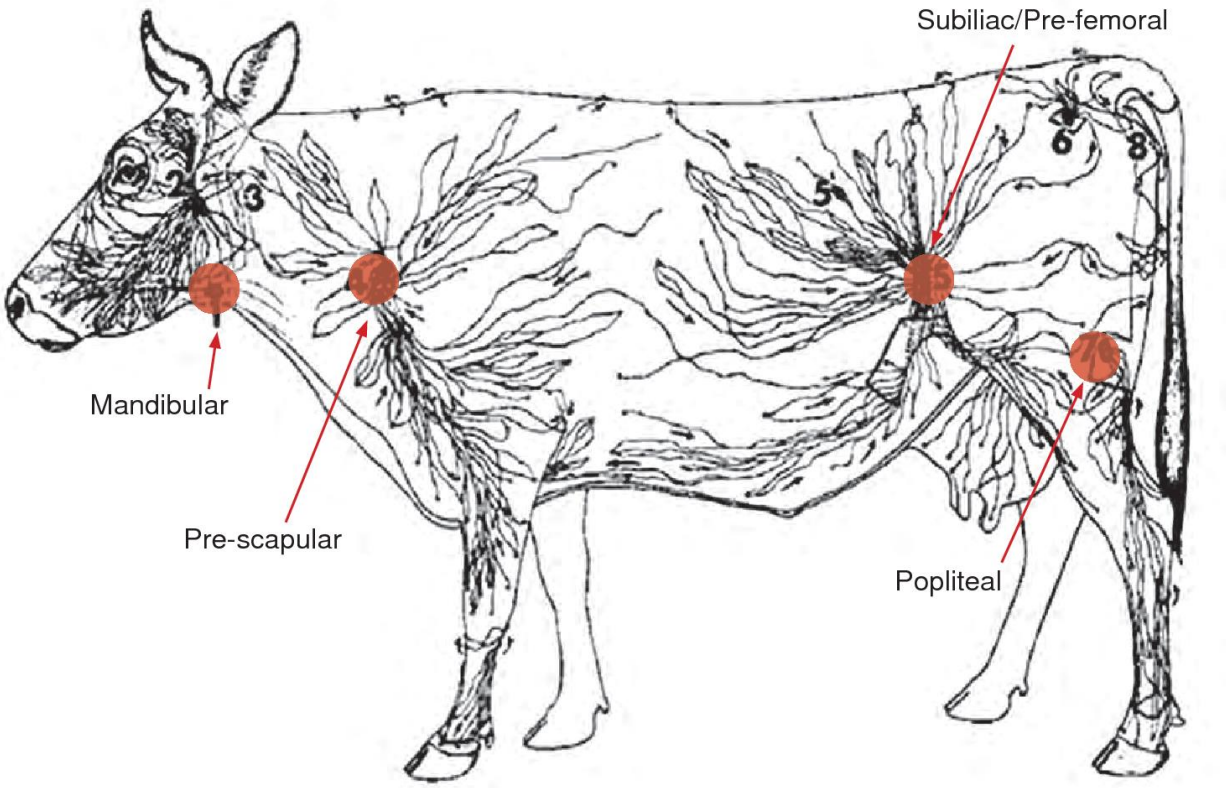
**Figure 1.1.** Prominent peripheral lymph nodes and their location on the body of a Cow (from Brichta-Harhay et al., (2020)).



# Chapter 2 - Evaluation of the effectiveness of a postbiotic product in reducing *Salmonella* prevalence in peripheral lymph nodes of cull dairy cattle

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## 2.1 Abstract

Cattle lymph nodes (LN) have been identified as a potential source of *Salmonella* in beef. Since post-harvest interventions cannot effectively control this route of contamination and the complete removal of PLN from beef carcasses is not practically feasible, there is a need for other means of mitigation. Dietary additives fed to cattle before harvest have shown promising results in the reduction of the prevalence and concentration of *Salmonella* in cattle LN. The objectives of this study were 1) to determine whether a larger scale or farm-level use of a commercially

available postbiotic product (Nutritek™ (Diamond V, Cedar Rapids, Iowa, U.S.)) compared to not feeding a postbiotic product (i.e., No-postbiotic) is associated with the reduction in the prevalence and concentration of *Salmonella* organisms in the subiliac LN of cull dairy cows harvested in two large U.S. commercial processing plants, across seasons (**Study 1**), 2) to determine associations between demographic and management characteristics, season, and plant/region with prevalence of *Salmonella* in bovine LN (**Study 1**), and 3) to provide a baseline estimate of the *Salmonella* LN prevalence and serovar diversity from cull dairy cattle from different U.S. regions over time (**Study 2**).

In collaboration with two commercial processing plants in the Southwestern (SW) and Northeastern (NE) regions of United States (U.S.), cull dairy cattle lots processed on the same week from dairy farms that fed the postbiotic or did not feed the postbiotic (No-postbiotic) were identified and sampled (**Study 1**). For the baseline prevalence study, LN samples from dairy farms supplying cattle to five processing plants in four U.S. regions (Upper Midwest (UMW), Midwest (MW), Northeast (NE) and Southwest (SW)) were collected (**Study 2**). Up to 20 LN were collected from each farm at least once every season, for both studies. Samples were analyzed for *Salmonella* by culture and quantitative PCR methods. Isolates were subjected to serotype identification via a classical serotyping and molecular typing, as well as to an antimicrobial susceptibility testing using the broth micro-dilution method.

Although a numerical reduction in prevalence was observed, the administration of the postbiotic product at pre-harvest was not effective at significantly reducing *Salmonella* LN prevalence in cull dairy cattle. *Salmonella* LN prevalence however, significantly varied by region/processing plant, where the SW region showed a higher prevalence than its NE counterpart. These findings were consistent with the baseline evaluation of *Salmonella* LN

prevalence in cull dairy cattle across different U.S. regions and seasons, where region and season were the main drivers of prevalence.

Within Study 1, dominant serotypes included Montevideo, Mbandaka, Muenster, Cerro, Meleagridis, and Anatum. The probability of isolating a *Salmonella* serotype did not significantly vary by feed additive status or region, however, it did vary by season. Between 0 and 34 isolates exhibited resistance to each antimicrobial, with streptomycin and ciprofloxacin showing the highest numbers of resistant isolates. There were seven isolates that were considered multi-drug resistant; one belonged to a sample from a postbiotic supplier from the NE region, whereas the remaining 6 isolates were from No-postbiotic samples, 5 from the SW and one from the NE region. In Study 2, dominant serotypes included Montevideo, Muenster, Mbandaka, Anatum, and Cerro. As with Study 1, the probability of isolating a *Salmonella* serotype did not significantly vary by region, but it did vary by season. For each antimicrobial, there were between 0 and 35 isolates that exhibited a resistant phenotype, with streptomycin showing the highest number of resistant isolates. There were four isolates that were considered multi-drug resistant; two belonged to a sample from the SW region, one from the UMW and one from the NE region.

The longitudinal nature of the data obtained from several processing plants and seasons enhanced our knowledge regarding the environmental and ecological factors that may be contributing to the carriage of *Salmonella* in cattle LN. Distinguishing risk factors that can enhance surveillance efforts can help processing plants manage problems before they become overwhelming, protect public health and the beef industry, and ensure food safety. Specifically, plant managers should streamline the application of pathogen control measures factors in specific

regions (e.g., Southwest) and seasons (e.g., winter), as these are consistently found to be important drivers of *Salmonella* LN prevalence.

Keywords: *Salmonella*, prevalence, subiliac LN, dairy cattle, feed additive, food safety

## 2.2 Introduction

There is currently a growing concern about *Salmonella* as several thousands of human illnesses per year in the U.S. have been attributed to food contamination by this pathogen (CDC, 2022). *Salmonella* enterica subspecies enterica, which is also a member of the *Enterobacteriaceae* family and closely related to *Escherichia coli*, has long been considered a pathogen of humans and animals (Brichta-Harhay et al., 2020). In humans, *Salmonella* organisms cause two general forms of disease following consumption of contaminated food/water or direct contact with infected animals; typhoidal salmonellosis, characterized by systemic disease and several clinical symptoms ranging from fever, cephalgia, myalgia, anorexia to severe weight loss and death if antibiotics are promptly not administered, and non-typhoidal salmonellosis, which constitutes a greater public health concern, with clinical signs ranging from fever to symptoms typical of the gastrointestinal tract (Brichta-Harhay et al., 2020; CDC, 2022; Grunberg, 2020).

As the number of human illness associated with other foodborne pathogens has decreased, *Salmonella* remains a persistent public health concern both in the U.S. and worldwide (Brichta-Harhay et al., 2020); according to Majowicz et al., (2010), approximately 94 million human illnesses worldwide each year have been attributed to salmonellosis, whereas CDC (2022) reported over 1.3 million illnesses, 26 thousand hospitalizations and over 400 deaths annually caused by *Salmonella* in the U.S. One of the potential sources of this pathogenic

bacterium in cattle, poultry and swine is the lymphatic system, specifically the LN (Brown et al., 2015; Edrington et al., 2013b & 2020). Lymph nodes function as a filtering mechanism to sequester bacteria, viruses, and other infectious agents for eventual destruction by lymphocytes (Buettner et al., 2012). However certain bacteria, such as *Salmonella*, frequently evade this process and instead survive within macrophages. The isolation of *Salmonella* organisms from cattle LN has been reported in several studies (Samuel et al., 1980, Sofos et al., 1999, Webb et al., 2017, Wottlin et al., 2022). Whereas some studies have concentrated on evaluating superficial LN (Edrington et al., 2020; Gragg et al., 2013a; Wottlin et al., 2022; Webb et al., 2017), others have streamlined *Salmonella* enterica contamination to mesenteric LN (Samuel et al., 1979, 1980 and 1981, Sofos et al., 1999), LN that are generally not included in ground beef as they are discarded during the evisceration process. Unlike the mesenteric LN, superficial LN such as the subiliac and cervical are located within the adipose tissue of muscle cuts (such as flanks and chucks, respectively) and are prone to be included in ground beef (Brichta-Harhay et al., 2020).

Presently there are several strategies, pre- and post-harvest, put forward to reduce the prevalence and concentration of *Salmonella* in ground beef (Brichta-Harhay et al., 2020). Of the several pre-harvest intervention strategies available (vaccination, biosecurity, animal washes and use of bacteriophages), there has been a growing interest in non-antimicrobial alternatives such as the use of feed additive, particularly a postbiotic product derived from the fermentation process of a yeast species, *Saccharomyces cerevisiae.* This product has been shown to have a beneficial effect at reducing enteric pathogens across multiple livestock species (Brewer et al., 2014; Feye et al., 2016b). This is primarily achieved via improvement of the health of the gastrointestinal tract (Jensen et al., 2008) and by facilitating immune body cells functions

(Jensen et al., 2007). Though previous studies have focused on its beneficial effects in animal production, recent studies have taken a turn to investigate its anti-pathogenic effects, as reported by Brewer et al. (2014) in a case study where this product had a significant reduction in the *Salmonella* load and virulence in the intestines and feces of pre-weaned dairy calves while boosting productivity. Similarly, Feye et al. (2016a), fed adult cattle this postbiotic product (formerly *S. cerevisiae* fermentation product) resulting in a decrease in the *Salmonella* LN colonization and fecal shedding as compared to their counterparts that did not receive the product. Also, in a recent study, Feye et al., (2019) found a significant reduction in the LN infiltration, invasiveness, and antibiotic resistance of *Salmonella* organisms in cattle and poultry fed the product.

Although limited, most data on the efficacy of feed additives to reduce the risk of *Salmonella* LN carriage in cattle have been obtained from pen-level randomized trials. Advantages of experimentally allocating study subjects include the ability to control potential confounders (both measured and unmeasured), which is achieved by randomization. Even though experimentally allocating study subjects through randomization is advantageous and leads to higher internal validity, controlling for potential biases implies a control over management practices, such as the type of facility (i.e., research feedlots), pen size (i.e., small pen size), and other factors (e.g., sorting), that often compromise the generalizability of the study, limiting the extrapolation of results to real-world situations. One of the reasons as to why results from pen-level studies may not be reproduced at a larger scale is likely due to the variability associated with farm environments. Furthermore, and very importantly - *Salmonella* can readily spread between pens and “herd immunity” effects can be important; thus, efficacy of interventions may be biased in pen-level studies (underestimating effects of interventions),

whereas larger scale or farm-level/feedlot applications of interventions may be much more effective.

Therefore, the objectives of this study were 1) to determine whether a larger scale or farm-level use of a commercially available postbiotic product compared to not feeding a postbiotic product (i.e., No-postbiotic) is associated with the reduction in the prevalence and concentration of *Salmonella* organisms in the subiliac LN of cull dairy cows harvested in two large U.S. commercial processing plants, across seasons (**Study 1**), 2) to determine associations between demographic and management characteristics, season, and plant/region with the prevalence of *Salmonella* in bovine LN (**Study 1**), and 3) to provide a baseline estimate of the *Salmonella* LN prevalence and serovar diversity from cull dairy cattle from different U.S. regions over time (**Study 2**).

## 2.3 Materials and methods

### 2.3.1 Study design and study population

**Study 1.** *Effectiveness of a Postbiotic product (POSTBIOTIC) in cull dairy cattle*. In this observational study, with the collaboration of two large commercial packing plants that process cull dairy cattle from surrounding states located in the Northeast (Wisconsin) and Southwest (Arizona) regions of the U.S., dairy farms ("suppliers") that routinely use the commercially available in-feed postbiotic product (Nutritek™ (Diamond V, Cedar Rapids, Iowa, U.S.)), and suppliers that do not administer any postbiotic product (No-postbiotic) were identified. Cattle from No-postbiotic suppliers were procured and processed in the same week and belonged to the same class of cattle (body weight and cattle type (dairy)) as the feed additive-supplier.

Criteria for selection of processing plants included plants, from the same meat packing company, that processed predominantly cull dairy cattle, and that procured cattle from postbiotic suppliers. Inclusion criteria for suppliers included dairy farms that process adult cull cattle; additionally, postbiotic suppliers had to administer the postbiotic product to cattle for at least 100 days before being sent to slaughter.

Once cattle lots from postbiotic and No-postbiotic suppliers were identified, up to 20 LN (one per cattle) per supplier were collected at least once in each season, across all seasons (winter (December to February), spring (March to May), summer (June to August) and fall (September to November)).

**Study 2.** *Baseline Salmonella prevalence in cull dairy cattle across regions and seasons.* Five large commercial processing plants that process cull dairy cattle located in the Upper Midwest (Michigan), Midwest (Nebraska), Northeast (Pennsylvania and Wisconsin), and the Southwest (Arizona) regions, all from the same meat packing company, were enrolled in the study. Each week, suppliers that process adult cull cattle were identified, and a subset of their cattle LN procured and processed. Up to 20 LN (one per cattle) per supplier were collected at least once each season, across all seasons (winter (December to February), spring (March to May), summer (June to August) and fall (September to November)) from all enrolled processing plants.

### 2.3.2 Sample size calculation

**Study 1.** A priori sample size estimates were computed assuming: 1) collecting up to 20 LN per supplier, 2) a mean *Salmonella* LN prevalence ranging between 15% and 30% among suppliers that do not administer the feed additive (No-postbiotic), 3) a reduction of 50% in

*Salmonella* LN prevalence among suppliers that administer the feed additive (postbiotic), 4) alpha (type I error) of 5%, 5) 80% power (type II error of 20%), and 6) an intraclass correlation coefficient of 8%. A minimum number of five suppliers per group, and per plant, a minimum of 15 suppliers per plant (~ 7 postbiotic and 8 No-postbiotic suppliers), for a minimum total of 30 suppliers across two plants, and a minimum total of 2,400 LN samples across four seasons were considered sufficient to achieve 80% statistical power and 95% confidence to detect expected differences in *Salmonella* prevalence between suppliers administering or not the postbiotic. Sample size calculations were performed in Stata® 17 (StataCorp LLC, College Station, Texas).

**Study 2.** A convenience sample of processing plants was selected for this study. The same number of LN samples to be collected per supplier and per sampling was chosen as per Study 1.

### 2.3.3 Lymph node collection and processing

Methods for collection, processing and laboratory analysis of LN were previously described by Cernicchiaro et al. (2016) and are shared between studies (Studies 1 and 2). Briefly, cattle supplied by selected dairy farms were identified according to their feed additive status, and peripheral (subiliac) LN along with the surrounding adipose tissue were removed by trained plant personnel in each sampling. Employees of the harvest facilities removed LN post-fabrication using knife-cuts which were performed according to protocols established by our team in consultation with the plants' Food Safety and Quality Assurance managers. After collection, samples were placed in pre-labeled Whirl-Pak (3M™, Maplewood, MN) sample collection bags and shipped in refrigerated containers overnight to the Pre-Harvest Food Safety

Laboratory at the College of Veterinary Medicine, Kansas State University (KSU-PHFSL) in Manhattan, Kansas, for processing and testing.

Samples were processed between 24 and 72 h of sampling. Upon arrival of the samples, the excess fat and fascia around the LN was trimmed using scissors or scalpels, LN were placed in petri-dishes and their individual weights recorded in grams. The trimmed LN were then subjected to surface sterilization in 70% ethanol for 2-3 s after which they were air-dried in sterile petri-dishes. The dried LN were then placed individually into Whirl-Pak bags and pulverized using a rubber mallet.

### 2.3.4 Laboratory analysis of lymph nodes

After LN were processed and contents placed in bags, 80 mL of tryptic soy broth (TSB) were added and hand-mixed within each bag for 30 s. For the pre-enrichment RT-PCR, 1 mL of the pre-enriched homogenate was transferred into a 2 mL microfuge tube, boiled for 10 m and centrifuged for 5 m at 9,300 X g. Samples were then stored at -20°C until ready for DNA extraction, whereas for quantification of *Salmonella*, DNA was extracted from pre-enrichment broth, from culture positive samples using the Geneclean DNA extraction kit (MP Biomedicals, solon, OH) and subjected to qPCR. The remaining TSB + LN homogenate was incubated at room temperature (25°C) for 2 h and then at 42°C for 12 h.

After incubating the samples at 42°C for 12 h, 10 mL of the enriched TSB culture were mixed with 90 mL of tetrathionate broth (TT broth) with iodine and incubated at 37°C for 18-24 h. The Whirl-Pak contents were then hand-mixed and 100 µL of enriched TT culture were removed and added to 5 mL Rappaport Vassiliadis broth (RV broth). Tubes were vortexed for 1 m and incubated at 42°C for 18-24 h. *Salmonella* cultures from the enriched RV broth, after

being vortexed for 1 m, were transferred onto Hektoen Enteric (HE) agar plates using sterile swabs; streak plating was completed on the HE agar plates for isolation using sterile inoculating loops after which the plates were incubated at 37°C for 18-24 h.

A maximum of three single presumptive *Salmonella* colonies (greenish blue colonies with black centers) were picked (labeled as A, B, and C) to blood agar and incubated at 37°C for 18-24 h. Colonies from blood agar were subjected to an agglutination test using *Salmonella* O antiserum on a microscope slide, and if positive, a PCR procedure targeting the *inv*A and *pag*C genes was performed for genus confirmation. Vials containing positive colonies were labeled (with the isolate ID and date of storage) and stored at -80°C.

### 2.3.5 Polymerase chain reaction (PCR) assay

The procedure for the PCR assay has been described in detail elsewhere (Bai et al., 2018). Briefly, each qPCR reaction was performed in a tube containing 20 µL total volume, which included 10 µL of 2 × iQ Multiplex Powermix (Bio-Rad, Hercules, CA), 1 µL of each (4) primer (10 pM/µL), 1 µL of each (2) probe (10 pM/µL), 2 µL of the extracted DNA and 2 µL of nuclease-free water. A positive and negative controls were included in each of the individual PCR experiments. Following sealing of the plates, they were then centrifuged for 1 min to ensure that unwanted bubbles remain settled at the top and not the bottom to avoid getting incorrect results. The plates were then placed in a CFX96 Touch Real-Time PCR after which reactions were run 10 min initial denaturation at 95°C followed by 45 cycles of 95°C for 15 s and 62°C for 50 s. The 62°C optimal annealing/extension temperature of the PCR assay was determined through a temperature gradient assay in a two-step PCR protocol in which the annealing and extension stages were combined. Correlation coefficients and PCR amplification efficiencies

were determined using the CFX Manager software (BioRad, Hercules CA). Samples were considered *Salmonella* positive when both quantification cycle values (Cq) for *inv*A and *pag*C were less than 38.

All available sequences of *inv*A and *pag*C genes from *Salmonella* enterica were downloaded from the GenBank website (https://www.ncbi.nlm.nih.gov/genbank/). Primers and probes were selected using the online PCR design tool, Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) (Untergasser et al., 2012). The *inv*A and *pag*C design was specific to *S. enterica*. FAM and MAX (VIC-equivalent) channels were selected for *inv*A and *pag*C respectively. Primers and probes were synthesized from Integrated DNA Technologies (Coralville, IA).

### 2.3.6 *Salmonella* serotyping and antimicrobial susceptibility testing

*Salmonella* isolates that were confirmed by PCR were streaked on tryptic soy agar (TSA) slants and incubated overnight at 37°C. Following the incubation period, the inoculated TSA slants were properly packaged and shipped to the National Veterinary Services Laboratory (NVSL) at Ames, Iowa for *Salmonella* serotyping. *Salmonellae* were typed via classical serotyping using polyvalent and single factor antisera to determine the O and H antigens and via molecular typing using the Luminex xMAP *Salmonella* serotyping assay (Fitzgerald et al., 2007; McQuiston et al., 2011).

The susceptibility of the isolates to 15 antimicrobial agents was determined using the broth micro-dilution method, which involves testing the growth of bacteria in a series of dilutions of different concentrations of antimicrobial agents. The minimum inhibitory concentrations (MIC) for each agent were determined using breakpoints established by the Clinical and Laboratory Standards Institute (CLSI, 2017). In cases where breakpoints were not

available, results were interpreted using criteria established by the National Antimicrobial Resistance Monitoring System (NARMS, 2019). Isolates were classified as susceptible, intermediate, or resistant depending on their MIC for each agent. Isolates that were resistant to three or more classes of antimicrobials were considered multi-drug resistant (MDR).

### 2.3.7 Questionnaire

As part of Study 1, a questionnaire, written in English, was designed and reviewed by all authors. Although pre-testing was not implemented in a subset of the study population, the questionnaire was reviewed by two experts in dairy practice and nutrition. Upon expert advice, the questions were refined, and a final version of the questionnaire generated. Closed- and open-ended questions were included to gather information on cattle type, cattle management, and production environment factors (e.g., cattle health, cattle source/origin, pen management, dietary and vaccination history, weather). The questionnaire was administered via a telephone or face-to-face interview to dairy farm managers by the processing plants' procurement teams. Digital copies of the questionnaires were sent to the Center for Outcomes Research and Epidemiology, at the College of Veterinary Medicine, Kansas State University (KSU-CORE) for data transcription, storage, and analyses. All identifiers were removed prior to data logging to maintain confidentiality. The questionnaire is available in Appendix 1.

### 2.3.8 Data analysis

**Study 1.** *Effectiveness of a postbiotic product in cull dairy cattle*. Frequency tables for prevalence and concentration of *Salmonella* were computed by feed additive status, season (and month), and region (processing plant). Similarly, the frequency of serotypes and of antimicrobial

resistant isolates was computed by feed additive status, season, and region. Initially, isolates were classified as susceptible, intermediate, or resistant depending on their MIC for each antimicrobial. For analysis purposes, both resistant and intermediate isolates were considered as resistant. Isolates that were resistant to three or more classes of antimicrobials were considered multi-drug resistant (MDR), and these results were described using frequency tables by season, region and feed additive status.

Multivariable mixed-effects logistic regression models were fitted to evaluate associations between feed additive status (postbiotic vs. No-postbiotic), season (summer, fall, winter, or spring) and region (Northeast, or Southwest), and two-way interaction terms between feed additive status and season, feed additive status and region, or season and region, with the within-supplier prevalence of *Salmonella* in subiliac LN. The dependent variable consisted of the number of *Salmonella* test-positive LN samples from each supplier identified in each sampling divided by the number of total LN sampled from each supplier in each sampling (events/trials). A binomial distribution, logit link and restricted pseudolikelihood estimation were fitted in a generalized linear mixed-effects model (GLMM). Region was included as a random intercept, depending on the fixed effects fitted, as well as a first-order autoregressive (ar(1)) covariance structure to account for repeated measures of farms (suppliers) nested within week of sampling (and within year), which was included in all models.

Associations between feed additive status (postbiotic or No-postbiotic), season (summer, fall, winter, or spring) and region (Northeast, or Southwest), and two-way interaction terms between feed additive status and season, feed additive and region, or season and region with the probability of isolating a *Salmonella* serotype were tested in multivariable mixed-effects multinomial logistic regression models. The dependent variable consisted of *Salmonella*

serotype, which was modeled as a nominal variable (4 levels; Montevideo, Muenster, Mbandaka, which were the three most frequent serotypes in this dataset, and other (for all other serotypes)). The category "other" was set as the baseline (referent) level. A GLMM was fitted with a multinomial distribution, glogit link, and residual pseudolikelihood estimation. A random intercept was included to account for the lack of independence among samples within suppliers.

Associations between feed additive status (postbiotic or No-postbiotic), season (summer, fall, winter, or spring) or region (Northeast, or Southwest), with the probability of *Salmonella* resistant isolates were tested in univariable mixed-effects logistic regression models. The dependent variables consisted of the probability of a *Salmonella* isolate being resistant to streptomycin, and the probability of a *Salmonella* isolate being resistant to ciprofloxacin. Only univariable models were attempted given the low effective sample size of these outcomes. Random intercepts for supplier, month within year, and region (if not included as fixed effect) were fitted in these models.

Unless stated, for all outcomes, univariable models were built first (testing one fixed effect at a time against the outcome), followed by a multivariable main effects model including the three fixed effects (feed additive status, region and season). Subsequently, each two-way interaction term was tested, one at a time, and only those with $P < 0.05$ were considered statistically significant and kept in the final multivariable models. Means, standard errors of the means and mean 95% confidence intervals were reported from mixed models' results (i.e., model-adjusted values that accounted for the other factors in the models). Data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC) using the Proc Glimmix package.

**Study 2.** *Baseline Salmonella prevalence in cull dairy cattle across regions and seasons.* Frequency tables for prevalence, concentration, serotype, antimicrobial resistance and multi-drug resistance results were computed by season (and month) and region. Statistical analyses of within-supplier *Salmonella* LN prevalence and probability of resistant isolates were implemented as described above, with the main difference being that no feed additive status effect was relevant in these models. Only a univariable model between season and the probability of isolating a *Salmonella* serotype was fitted, including a random intercept for supplier within region, as described for Study 1.

*Salmonella* concentration data, prevalence of antimicrobial resistance or of multi-drug resistance were not analyzed and presented descriptively only, given the low number of enumerable or resistant isolates, respectively.

### 2.3.9 Blinding

For study 1, sampling plant personnel, laboratory personnel, and the data analyst were blinded to feed additive status of the suppliers throughout the duration of the study.

## 2.4 Results

### 2.4.1 Lymph sample collection and *Salmonella* crude prevalence

**Study 1.** A total of 2,816 LN samples were collected from cull dairy cattle presented for harvest across feed additive status, regions/processing plants and seasons, over a two-year sampling period (May 28, 2021, to July 19, 2023).

Between 1 and 24 LN were collected per supplier, and per sampling, in each season, from a total of 89 suppliers across the two processing plants (72 from the NE region/plant and 17 from the SW region/plant). Therefore, between 652 and 791 LN were collected each season.

The overall crude *Salmonella* prevalence was 13.9% (391/2,811). *Salmonella* LN prevalence in postbiotic farms (11.8%, 100/847) and the No-postbiotic farms (14.8%, 291/1,969) across all seasons and regions is depicted in Table 1. *Salmonella* prevalence was 10.6% (84/791) in summer, 12.8% (84/656) in fall, 20.0% (131/652) in winter, and 12.8% (92/717) in spring (Table 2.1).

Across both feed additive status and all seasons, the SW region had a *Salmonella* prevalence of 20.3% (281/1,384) compared to a 7.7% (110/1,432) for the NE region. Table 2.1 depicts the proportion and percentage of *Salmonella* test-positive LN of cull dairy cattle by sampling season and month, region, and feed additive status.

**Study 2.** A total of 2,231 samples were collected from September 29, 2022, to July 27, 2023, from 122 suppliers (14 from Michigan, 32 from Pennsylvania, 47 from Wisconsin, 20 from Nebraska, and 13 from Arizona) (Table 2.11). Of note, is the fact that four suppliers did not assign a specific location/name and they supplied cattle to multiple plants, as such the sum of the suppliers by region exceeds the total number (n = 122). Between 1 and 24 LN were collected per supplier, and per sampling, in each season, and between 8 and 32 suppliers were recruited per plant.

The overall crude *Salmonella* LN prevalence was 13.8% (307/2,231). *Salmonella* LN prevalence by region was as follows: 11.0% (78/710) in the UMW, 9.6% (25/260) in the MW, 8.2% (39/473) in the NE, and 20.9% (165/788) in the SW region. *Salmonella* prevalence was 11.5% (77/672) in summer, 11.9% (28/235) in fall, 19.6% (105/537) in winter, and 12.3%

(97/787) in spring. Table 2.11 depicts the proportion and percentage of *Salmonella* test-positive LN of cull dairy cattle by season, month, and region.

### 2.4.2 Effectiveness of the postbiotic and model-adjusted *Salmonella* LN prevalence

**Study 1.** The within-supplier *Salmonella* LN prevalence did not significantly ($P > 0.05$) differ by feed additive status, in univariable or multivariable analyses.

Based on univariable models, neither feed additive status nor season were statistically significant, whereas region was significantly associated ($P < 0.001$) with the mean within-supplier *Salmonella* LN prevalence (Table 2.5).

When building the final multivariable model, none of the tested two-way interaction terms were significant ($P > 0.05$). Hence, the final model included fixed effects for feed additive status and season, which were forced into the model (being the primary explanatory variable of interest and *a priori* confounder, respectively), and for region. Although not significantly different ($P = 0.485$), the within-supplier *Salmonella* LN prevalence among postbiotic suppliers was 11.65%, and 13.10% among No-postbiotic suppliers. Yet not statistically significantly ($P = 0.319$), the within-supplier *Salmonella* LN prevalence was higher during winter, followed by fall, summer and spring seasons. The SW region had a significantly ($P < 0.001$) higher within-pen *Salmonella* LN prevalence (20.01%) than the NE (7.80%) region (Table 2.6).

**Study 2.** Based on univariable models, the mean within-supplier *Salmonella* LN prevalence did not significantly differ by season ($P = 0.217$), but it did by region ($P < 0.001$). Specifically, the within-supplier *Salmonella* LN prevalence was significantly higher in the SW region than the NE, and in the SW than the UMW region (Table 2.15).

The final multivariable model included fixed effects for season, region and a two-way interaction term between season and region ($P = 0.027$). The within-supplier LN prevalence of *Salmonella* was highest for the SW region during the fall season (34.8%) and lowest for the NE region during the fall months (1.6%) (Table 2.16). Specifically, the within-supplier *Salmonella* LN prevalence was significantly higher in the SW region during winter than in the UMW in the spring season ($P = 0.026$)**.** None of the other contrasts were statistically significant ($P > 0.05$).

### 2.4.3 *Salmonella* concentration

**Study 1.** Overall, *Salmonella* concentration was 0.04% (1 quantifiable sample / 2,816 of LN samples tested). The only quantifiable sample was observed during the summer season from a No-postbiotic supplier of the NE region and had a concentration of $2.22 \times 10^5$ ($5.35 \log_{10}$) CFU/g LN.

**Study 2.** Overall *Salmonella* LN concentration was 0.1% (3 quantifiable samples / 2,231 samples tested). Two of the quantifiable samples were observed during the spring and summer seasons of the UMW region and had concentrations of $4.61 \times 10^4$ ($4.66 \log_{10}$) CFU/g LN, and $8.18 \times 10^5$ ($5.91 \log_{10}$) CFU/g LN, respectively, whereas the remaining quantifiable sample was observed during the summer season of the NE region and had a concentration of $2.22 \times 10^5$ ($5.35 \log_{10}$) CFU/g LN.

### 2.4.4 *Salmonella* serotyping

**Study 1.** Out of 391 total isolates serotyped, their distribution between regions was as follows; 110 (28.1%) from the NE and 281 (71.9%) from the SW region. Dominant serotypes across all the plants/regions and suppliers include Montevideo (27.1%), Mbandaka (16.4%),

Muenster (14.1%), Cerro (10.5%), Meleagridis (5.6%), and Anatum (4.9%). Other serotypes of human importance identified in this study included Typhimurium (5/391, 1.3%) and Newport (2/391, 0.8%). Table 2.2 shows the distribution of *Salmonella* serotypes isolated from the subiliac LN of cull dairy cattle by region and feed additive status.

Based on univariable models, neither feed additive status nor region were statistically significant, whereas season was significantly associated ($P = 0.002$) with the probability of isolating a *Salmonella* serotype (Table 2.7).

The final multivariable model included fixed effects for feed additive status and region, which were forced into the model (being the primary explanatory variable of interest and *a priori* confounder, respectively), and for season. Although not significantly different ($P = 0.385$), the mean probability of isolating serotype Montevideo, Muenster or Mbandaka vs. Other serotypes, was lower in samples from postbiotic than from No-postbiotic suppliers (Table 8). Also, not statistically significantly ($P = 0.362$), the probability of isolating serotype Montevideo, Muenster or Mbandaka vs. Other serotypes, was higher in samples from the SW than the NE region. Lastly, the probability of isolating a *Salmonella* serotype significantly varied by season ($P = 0.002$); the probability of isolating serotype Montevideo vs Other serotypes was higher in fall than in winter ($P = 0.004$), and in spring than in winter ($P = 0.035$), and for Muenster vs. Other serotypes, it was higher in winter than in summer ($P = 0.028$) (Table 2.8).

**Study 2.** Out of 307 total isolates serotyped, the number of isolates per region was as follows: 78 from UMW, 25 from the MW, 165 from the SW and 39 from the NE region. Dominant serotypes across all the regions and seasons included Montevideo (19.2%), Muenster (17.3%), Mbandaka (15.3%), Anatum (12.4%), and Cerro (9.4%). Another serotype of human importance which was identified in this study included Typhimurium (9/307, 2.9%) (Table 2.12).

Based on univariable analysis, the probability of isolating serotype Montevideo vs Other serotypes was higher in fall than in summer ($P = 0.011$), and in fall than in winter ($P = 0.004$), for Muenster vs. Other serotypes, it was higher in fall than in spring ($P = 0.009$), summer than in spring ($P = 0.004$), and in winter than in spring ($P = 0.015$) (Table 2.17).

### 2.4.5 Antimicrobial susceptibility testing

**Study 1.** A total of 391 *Salmonella* isolates were subjected to antimicrobial susceptibility testing. Antimicrobials used include Augmentin (Amoxicillin and Clavulanic acid), Ampicillin, Azithromycin, Cefoxitin, Ceftiofur, Ceftriaxone, Chloramphenicol, Ciprofloxacin, Gentamicin, Meropenem, Nalidixic acid, Streptomycin, Tetracycline, and Sulfamethoxazole-Trimethoprim.

Of the 391 isolates that were subjected to antimicrobial susceptibility testing, approximately 18% (69/391, 17.6%) showed resistance to at least one antimicrobial; seventeen belonged to isolates from postbiotic and 52 to No-postbiotic cattle.

For each antimicrobial, there were between 0 and 34 isolates that exhibited a resistant phenotype. The antimicrobial showing the highest number of resistant isolates was streptomycin with 34 (34/391, 8.7%) resistant isolates, followed by ciprofloxacin (26/391, 6.7%) (Table 2.3).

Based on univariable models, there was no significant difference in the probability of isolating streptomycin and ciprofloxacin-resistant *Salmonella* isolates among feed additive status (postbiotic and No-postbiotic), season (Summer, Fall, Winter, and Spring), or regions (NE and SW) (Table 2.9).

There were seven (7/391, 1.8%) isolates that were considered multi-drug resistant and belonged to serotype Newport (n = 2), Typhimurium (n = 1), Kentucky (n = 1), Montevideo (n = 1), Anatum (n = 1), and Oranienburg (n = 1) (Table 4). One of the MDR isolates belonged to a

sample from a postbiotic supplier from the NE region, whereas the remaining six isolates were from No-postbiotic samples, five from the SW and one from the NE region.

Table 2.3 describes the proportion of resistant isolates to each antimicrobial and table 2.4 the number of multi-drug resistant isolates by season, region and feed additive status.

**Study 2.** A total of 307 positive *Salmonella* isolates were subjected to antimicrobial susceptibility testing. Antimicrobials used include Augmentin (Amoxicillin and Clavulanic acid), Ampicillin, Azithromycin, Cefoxitin, Ceftiofur, Ceftriaxone, Chloramphenicol, Ciprofloxacin, Gentamicin, Meropenem, Nalidixic acid, Streptomycin, Tetracycline, and Sulfamethoxazole-Trimethoprim.

Of the 307 isolates that were subjected to antimicrobial susceptibility testing, 165 (53.8%) belonged to LN from the SW region, 78 (25.4%) from the UMW, 39 (12.7%) from the NE, and 25 (8.1%) from the MW region (Table 2.13). Approximately 19% (28/307, 18.6%) of the isolates exhibited resistance to at least one antimicrobial.

For each antimicrobial, there were between 0 and 35 isolates that exhibited a resistant phenotype. The antimicrobial showing the highest number of resistant isolates was streptomycin with 35 (35/307, 11.4%) (Table 2.13).

Based on univariable analyses, there was no significant difference in the probability of isolating streptomycin-resistant *Salmonella* isolates among seasons (summer, fall, winter, and spring) or regions (NE, SW, MW, and UMW) (Table 2.18).

There were four (4/307, 1.3%) isolates that were considered multi-drug resistant and belonged to serotype Typhimurium (n = 1), Anatum (n = 1), Dublin (n = 1), and Oranienburg (n = 1). Two of the MDR isolates belonged to a sample from the SW region, one from the UMW, and one from the NE region (Table 2.14).

Table 2.13 describes the proportion of resistant isolates to each antimicrobial and table 2.14 the number of multi-drug resistant isolates by season and region.

### 2.4.6 Questionnaire

**Study 1.** Out of the 89 suppliers enrolled in Objective 1, we only obtained questionnaire responses from five of them, all belonging to No-postbiotic farms, which resulted in a 5.6% response rate. Table 2.10 depicts the responses of the five questionnaires gathered.

## 2.5 Discussion

Despite hide-to-carcass pathogen reduction interventions having successfully decreased surface contamination of *Salmonella*, contamination of ground beef and incidence of human disease associated with this bacterium have not declined. Peripheral LN are protected from chemical and thermal antimicrobial carcass surface treatments, and if not excised, they can be incorporated into ground beef and be a source of *Salmonella* contamination during processing. Because of the significant challenges associated with addressing LN *Salmonella* at the post-harvest phase, many within the beef industry strongly believe that a pre-harvest approach is necessary to mitigate contamination risks and contribute towards production of safe beef.

There are a few commercially available pre-harvest interventions that could be used to mitigate *Salmonella* in cattle. Although limited, most data on the efficacy of feed additives (and other pre-harvest interventions) to reduce the risk of *Salmonella* LN carriage in cattle have been obtained from pen-level randomized trials and not from observational studies evaluating on-farm large-scale or farm-level application of interventions.

The present study evaluated the effectiveness of a commercially available feed additive on reducing *Salmonella* prevalence in LN of cull dairy cattle under “real-world” conditions. We

quantified how much reduction in *Salmonella* prevalence occurred when producers implemented this pre-harvest intervention. Similarly, this study aimed to provide an estimate of the baseline prevalence of *Salmonella* and of serovar diversity in LN of cull dairy cattle from across different U.S. regions and seasons.

Although *Salmonella* can be recovered from several PLN, gastrointestinal tract-associated LN (e.g., mesenteric) are discarded during the evisceration process and, thus, do not pose a direct food safety hazard. We chose to sample subiliac LN, because they have greater potential to be incorporated into ground beef products due to their anatomical location residing in adipose tissues near important muscle cuts, and because they are more accessible for sampling during harvest. Similarly, we chose to assess *Salmonella* in LN of cull dairy cattle, given that this type of cattle contributes 19% of the 12.4 billion kg of commercial beef produced in the U.S. (ERS, 2019), which is mainly fabricated into ground beef.

Even though it showed a numerical reduction in prevalence, the larger scale or farm-level administration of a postbiotic product at pre-harvest did not significantly reduce *Salmonella* prevalence in LN of cull dairy cattle. Previous studies have looked at the effectiveness of postbiotic against *Salmonella* organisms (Brewer et al., 2014, Feye et al., 2019 and Price et al., 2010). Whereas Price et al., (2010) observed that inclusion of this product tended to increase *Salmonella* shedding in the feces, Brewer et al., (2014) and Feye et al., (2019) reported a reduction in the *Salmonella* intestinal colonization, and LN infiltration and invasiveness, respectively. It is important to note that these studies were experimental, resulting in higher internal validity but likely compromising external validity. In contrast to experimental studies, our study followed an observational design, where no control of the dose of the product, other dietary components in the feed or farm management practices was implemented. Therefore, it is

very likely that these or other unmeasured factors could have affected or reduced the effectiveness of this product or provided additional variability, preventing us from detecting a difference in *Salmonella* LN prevalence between the postbiotic and No-postbiotic farms. It is also worth mentioning that, although a 100 days feeding period of the product prior to harvest was established as an inclusion criterion during the design of the study, we were not able to corroborate whether all postbiotic suppliers adhered to the protocol; lower or variable duration of administration could also have had a role on the reduced effectiveness of the product. Another aspect to consider is the mechanism of action of postbiotic. While this is not still well understood, some studies suggest that it functions through the competitive exclusion mechanism, altering the microbiome in the gastrointestinal tract, and preventing the survival and subsequent uptake by the lymphatic system (Feye et al., 2019; Park et al., 2017; Rubinelli et al., 2016). Others speculate that this feed additive helps boost the immune system through the complement pathway, hence, reducing the invasiveness, survival, and pathogenic activities of *Salmonella* organisms (Feye et al., 2016, 2019; Nelson et al., 2020). However, it is essential to conduct additional studies to gain a more comprehensive understanding of the specific mechanism and the conditions under which this product is most effective in reducing *Salmonella* prevalence in cattle.

Although it differed by region, the overall mean within-supplier *Salmonella* LN prevalence was approximately 14%, which was lower than anticipated. *Salmonella* LN prevalence significantly varied by region/processing plant, where the SW region showed a higher prevalence than its NE counterpart. These findings were consistent with the baseline evaluation of *Salmonella* LN prevalence in cull dairy cattle across different U.S. regions and seasons (Study 2), where region/processing plant played also a significant effect on *Salmonella* LN prevalence,

although it also depended on the season. Again, the SW region showed the highest *Salmonella* LN prevalence, especially in the fall and winter months. Not only is region a direct proxy for processing plant in this study, as only one plant (or two for the NE region in Study 2) was included in each region, but also for environmental, climatic, management, and dietary factors, among others. It is worth noting that other studies have reported significant associations between *Salmonella* prevalence and environmental temperature variations (Gragg et al., 2013a, Levent et al., 2019; Nickelson et al., 2019, Stephen and Barnett, 2016, Webb et al. 2017, Wottlin et al., 2022). For example, Stephen and Barnett (2016) conducted more in-depth research on the association between temperature change and the prevalence of *Salmonella* in humans, identifying a 45.5% increase in *Salmonella* cases for every 5°C increase in environmental temperature. It has also been shown that heat stress reduces the integrity of tight junctions in the intestines (Koch et al., 2019), which may potentially explain the increase in *Salmonella* LN prevalence in warmer environments. However, when looking at *Salmonella* carriage in LN, temperature or other weather factors may not be as relevant as other elements that change based on seasonality such as management, diet, or physiological events that may impact infection and subsequent *Salmonella* LN harborage. Previous studies have also indicated a connection between the seasonality of vector presence or abundance and the prevalence of *Salmonella*. In these studies, it was observed that higher vector presence led to an increase in cases of salmonellosis due to their interactions with host animals (Olafson et al., 2016; Taylor et al., 2012; Xu et al., 2018). Other reports have also linked variations in *Salmonella* prevalence in cattle to prevalence in the supplier farm or plant environment (Nickodem et al., 2023; Villarreal-Silva et al., 2016). However, as our study was observational in nature and conducted at post-harvest, there were no

opportunities to measure vector presence and collect environmental samples at the farm, and collecting environmental samples at the plant was outside the scope of the study.

Another important aspect to consider is the concentration of *Salmonella* within the PLN of cattle. While there were no significant differences in prevalence estimates between farms using the postbiotic and those without it, examining differences in concentrations could have provided valuable insights into the effectiveness of the feed additive, especially when considering variations across seasons and regions. However, across both Study 1 and 2, only three samples yielded quantifiable *Salmonella* concentrations. This limited number of quantifiable samples contrasts with previous studies that reported variations in *Salmonella* concentrations across multiple sampled LN in different seasons and regions (Cernicchiaro et al., 2016; Gragg et al., 2013a; Webb et al., 2017; Wottlin et al., 2022). For instance, Cernicchiaro et al. (2016) found approximately 70% of LN samples to be quantifiable, while Gragg et al. (2013) reported that 58.8% of quantifiable nodes contained *Salmonella* concentrations ranging from 0.1 to 1.8 log10 CFU/g, and about 41% carried higher levels ranging from 1.9 to 3.8 log10 CFU/g. It is worth noting that these studies employed a more sensitive quantification method (*Enterobacteriaceae* count plates), whereas our quantification procedure relied solely on quantitative PCR with a detection limit of 4 $\log_{10}$ CFU/g. This limited our ability to quantify *Salmonella* isolates at lower concentrations.

Montevideo, Mbandaka and Muenster were the most frequently isolated serotypes, and their presence significantly varied by season. The dominant serotypes, however, are typically not linked to laboratory-confirmed cases of salmonellosis in humans. When they are associated, it is often from other sources such as spices, cheese, or poultry products (Webb et al., 2017). Other serotypes identified in this study included Typhimurium and Newport, which have been

frequently implicated in human salmonellosis and are usually multi-drug resistant (CDC, 2022). Consistent with other studies, these isolates were less frequently isolated from LN in our study (Webb et al., 2017 and Gragg et al., 2013a, b). However, these serotypes, even in low numbers are more dangerous and have been linked to a number of ground beef related outbreaks over the years (Schneider et al., 2011, CDC, 2012, 2013). Interestingly, the probability of isolating a *Salmonella* serotype did not significantly vary by feed additive status or region, however, it did vary by season. This is consistent with previous literature that have reported on seasonal changes affecting serotype diversity in dairy cattle, where serotypes isolated during the Summer were absent during Winter sampling , and only infrequently encountered in the subsequent Summer (Edrington et al., 2008, 2004). However, it is important to note that the serotyping results from this study may not provide a full representation of the possible serovars present as only one colony was selected to represent an LN sample. This means that the culture methodology also plays a major role in serotype recovery (Edrington and Brown, 2022), and so caution should be taken when interpreting serotype results.

Approximately 2% of isolates exhibited multi-drug resistance (MDR) in both studies. Although not in LN samples, previous studies have reported 5 to 10% occurrence of MDR *Salmonella* in fecal samples from cull dairy cattle (Loneragan et al., 2012; Rodriguez-Rivera et al., 2016). According to Gragg et al., (2013a), antimicrobial susceptibility phenotyping showed that most of the *Salmonella* isolated (86%) were susceptible to all antimicrobials tested; however, MDR *Salmonella* was observed in a higher proportion of isolates from cull cattle LN than in LN of feedlot cattle. Though overall isolation of MDR *Salmonella* was rare in a previous study (Wottlin et al., 2022), the greatest proportion came from cull beef and dairy cattle. With cull animals, it is possible that antimicrobial therapy was administered to the animals prior to

culling (Wottlin et al., 2022). Additionally, the increased age of this cattle type compared to feedlot and grass-fed cattle could provide more opportunities for them to receive antimicrobial therapy throughout life and therefore a greater prospect to develop resistance (Wottlin et al., 2022). It is also possible that the prevalence of MDR isolates may increase from the time cattle are transported from the farms to the processing plants; Schmidt et al., (2015) reported 0.5% prevalence of 3rd generation cephalosporin-resistant *Salmonella* in fecal samples collected at a feedlot, but that number increased to 1.6% at the time of slaughter.

While the majority of the isolates (up to 81%) were pan-susceptible to all the antimicrobial agents tested in this study, up to 19% exhibited resistance to at least one antimicrobial, predominantly to an aminoglycoside (i.e., streptomycin) or a quinolone (i.e., ciprofloxacin), with the latter being a clinically important antimicrobial drug class in human medicine. However, the resistance status of *Salmonella* isolates to these antimicrobials did not vary significantly among feed additive status (study 1), season and region (study 1 and 2). Recommended treatment options for salmonellosis include beta-lactams (i.e., penicillin or third generation cephalosporins) or fluoroquinolones (not recommended for children) — and alternative treatment recommendations include sulfamethoxazole-trimethoprim or azithromycin (Webb et al., 2017). In Webb's study, 14.3% of isolates were resistant to at least one of the antimicrobial classes recommended for treatment of life-threatening salmonellosis. Also, worth noting, 1.7% of the isolates were resistant to both beta-lactams and fluoroquinolones (Webb et al., 2017). However, in our studies, about 9% of the isolates demonstrated resistance to at least one of the antimicrobial classes recommended for human salmonellosis treatment while up to 2% showed resistance to beta-lactams and fluoroquinolones. It is also important to note that the presence of resistant isolates can also be linked to the consistent level of *Salmonella* prevalence

in ground beef over the years. For instance, recent findings from the Division of Foodborne, Waterborne, and Environmental Diseases (DFWED) indicate that since 2017, three distinct outbreaks of *Salmonella* tied to ground beef have been identified. Remarkably, these outbreaks have resulted in as many human illnesses and hospitalizations as the cumulative outbreaks observed over the preceding 36 years. This concerning trend could potentially be attributed to the presence of *Salmonella* serotypes resistant to interventions, and the contamination of ground beef due to dairy cow involvement (CDC, 2020).

Study limitations include enrolling fewer postbiotic suppliers than anticipated as part of Study 1. Originally, 12 enrolled dairy farms were considered postbiotic based on their historical use of the product. However, during the study period, five of those farms either discontinued their use of the product or reported not using the product at all, reducing considerably the number of dairy suppliers that administered the postbiotic product. Conversely, we obtained LN samples from 77 No-postbiotic dairy suppliers, which surpassed the number of suppliers needed for our study (n = 30). Unfortunately, a very low response to the questionnaire (5.6%), coupled with the fact that only managers from No-postbiotic farms responded, prevented us from implementing a risk factor analysis and disentangling potential associations between demographic, management, and dietary factors that may explain the differences in *Salmonella* LN prevalence across regions, seasons, and the effect of postbiotic administration.

Beef safety requires a comprehensive farm-to-plate approach with a commitment from all parties to reduce the burden of *Salmonella* in cattle as they enter the food chain and to ensure the safety of our beef. That includes the utilization by farmers of pre-harvest interventions in addition to good management practices, the risk-based application of pathogen control measures

factors by food processors/plants in specific regions and seasons, as well as the adoption of safe food preparation procedures by consumers.

The information obtained in this study can assist the beef industry by providing risk-based data that is immediately relevant to adoption of in-plant strategies for reducing *Salmonella* in beef and enables decision-makers to more appropriately consider the costs and benefits of this pre-harvest intervention. Furthermore, the longitudinal nature of the data obtained from different plants and seasons enhanced our knowledge regarding the environmental and ecological factors that may be contributing to the carriage of *Salmonella* in cattle LN. Similarly, generating data on frequency and distribution, amount and sources of variability associated with prevalence, serotype diversity and antimicrobial resistance of *Salmonella* in LN can improve the industry's understanding of the potential burden of *Salmonella* in trim and ground beef products. As *Salmonella* outbreaks have a devastating effect on public health and the beef industry, identifying effective mitigations to reduce *Salmonella* carriage in LN and distinguishing risk factors that can enhance surveillance efforts, will help processing plants manage problems before they become overwhelming, protect public health and the beef industry, and ensure food safety.

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Table 2.1. Study 1: Proportion and percentage of *Salmonella* test-positive lymph nodes of cull dairy cattle by season (and year), month, region, and feed additive status.

| Season (year) | Month | Region | Feed additive status | Proportion positives (%) |
|---|---|---|---|---|
| Summer (2021) | June | Northeast | Postbiotic | 2/31 (6.5) |
| | | | No-postbiotic | 4/90 (4.4) |
| | | Southwest | Postbiotic | 0/19 (0) |
| | | | No-postbiotic | 8/20 (40) |
| | July | Northeast | Postbiotic | 4/20 (20.0) |
| | | | No-postbiotic | 9/47 (19.1) |
| | | Southwest | Postbiotic | 0/18 (0) |
| | | | No-postbiotic | 1/17 (5.9) |
| | August | Northeast | Postbiotic | 0/0 (0) |
| | | | No-postbiotic | 1/20 (5.0) |
| | | Southwest | Postbiotic | 0/19 (0) |
| | | | No-postbiotic | 14/59 (23.7) |
| Fall (2021) | September | Northeast | Postbiotic | 4/23 (17.4) |
| | | | No-postbiotic | 0/22 (0) |
| | | Southwest | Postbiotic | 0/0 (0) |
| | | | No-postbiotic | 16/76 (21.1) |
| | October | Northeast | Postbiotic | 3/27 (11.1) |
| | | | No-postbiotic | 0/67 (0) |
| | | Southwest | Postbiotic | 0/20 (0) |
| | | | No-postbiotic | 0/17 (0) |
| | November | Northeast | Postbiotic | 1/26 (3.8) |
| | | | No-postbiotic | 2/26 (7.7) |
| | | Southwest | Postbiotic | 1/19 (5.3) |
| | | | No-postbiotic | 0/17 (0) |
| Winter (2021-22) | December | Northeast | Postbiotic | 3/19 (15.8) |
| | | | No-postbiotic | 2/32 (6.3) |
| | | Southwest | Postbiotic | 0/19 (0) |
| | | | No-postbiotic | 1/14 (7.1) |

| | | | | |
|---|---|---|---|---|
| | January | Northeast | Postbiotic | 0/8 (0) |
| | | | No-postbiotic | 0/11 (0) |
| | | Southwest | Postbiotic | 0/0 (0) |
| | | | No-postbiotic | 6/38 (15.8) |
| Spring (2022) | March | Northeast | Postbiotic | 0/0 (0) |
| | | | No-postbiotic | 1/28 (3.6) |
| | | Southwest | Postbiotic | 0/0 (0) |
| | | | No-postbiotic | 9/109 (8.3) |
| | April | Northeast | Postbiotic | 4/42 (9.5) |
| | | | No-postbiotic | 16/80 (20.0) |
| | | Southwest | Postbiotic | 0/0 (0) |
| | | | No-postbiotic | 0/0 (0) |
| | May | Northeast | Postbiotic | 2/25 (8.0) |
| | | | No-postbiotic | 0/27 (0) |
| | | Southwest | Postbiotic | 5/16 (31.3) |
| | | | No-postbiotic | 0/19 (0) |
| Summer (2022) | June | Northeast | Postbiotic | 1/51 (2.0) |
| | | | No-postbiotic | 3/93 (3.2) |
| | | Southwest | Postbiotic | 0/0 (0) |
| | | | No-postbiotic | 0/0 (0) |
| | July | Northeast | Postbiotic | 1/43 (2.3) |
| | | | No-postbiotic | 1/58 (1.7) |
| | | Southwest | Postbiotic | 0/0 (0) |
| | | | No-postbiotic | 0/0 (0) |
| | August | Northeast | Postbiotic | 0/10 (0) |
| | | | No-postbiotic | 3/24 (12.5) |
| | | Southwest | Postbiotic | 0/0 (0) |
| | | | No-postbiotic | 0/0 (0) |
| Fall (2022) | September | Northeast | Postbiotic | 0/5 (0) |
| | | | No-postbiotic | 0/17 (0) |
| | | Southwest | Postbiotic | 17/49 (34.7) |

| | | | | |
|---|---|---|---|---|
| | | | No-postbiotic | 15/54 (27.8) |
| | October | Northeast | Postbiotic | 2/39 (5.1) |
| | | | No-postbiotic | 0/40 (0) |
| | | Southwest | Postbiotic | 7/18 (38.9) |
| | | | No-postbiotic | 6/11 (54.5) |
| | November | Northeast | Postbiotic | 1/22 (4.5) |
| | | | No-postbiotic | 1/23 (4.3) |
| | | Southwest | Postbiotic | 2/19 (10.5) |
| | | | No-postbiotic | 6/19 (31.6) |
| Winter (2022-23) | December | Northeast | Postbiotic | 0/8 (0) |
| | | | No-postbiotic | 2/26 (7.7) |
| | | Southwest | Postbiotic | 1/20 (5.0) |
| | | | No-postbiotic | 21/57 (36.8) |
| | January | Northeast | Postbiotic | 3/7 (42.9) |
| | | | No-postbiotic | 5/49 (10.2) |
| | | Southwest | Postbiotic | 14/20 (70.0) |
| | | | No-postbiotic | 33/132 (25.0) |
| | February | Northeast | Postbiotic | 3/37 (8.1) |
| | | | No-postbiotic | 4/36 (11.1) |
| | | Southwest | Postbiotic | 7/40 (17.5) |
| | | | No-postbiotic | 25/79 (31.6) |
| Spring (2023) | March | Northeast | Postbiotic | 0/0 (0) |
| | | | No-postbiotic | 0/0 (0) |
| | | Southwest | Postbiotic | 3/35 (8.6) |
| | | | No-postbiotic | 17/78 (21.8) |
| | April | Northeast | Postbiotic | 2/17 (11.8) |
| | | | No-postbiotic | 1/17 (5.9) |
| | | Southwest | Postbiotic | 0/0 (0) |
| | | | No-postbiotic | 13/80 (16.3) |
| | May | Northeast | Postbiotic | 0/6 (0) |
| | | | No-postbiotic | 1/27 (3.7) |

| | | | | |
|---|---|---|---|---|
| | | Southwest | Postbiotic | 0/0 (0) |
| | | | No-postbiotic | 17/79 (21.5) |
| Summer (2023) | June | Northeast | Postbiotic | 2/12 (16.7) |
| | | | No-postbiotic | 5/12 (41.7) |
| | | Southwest | Postbiotic | 0/0 (0) |
| | | | No-postbiotic | 16/78 (20.5) |
| | July | Northeast | Postbiotic | 4/22 (18.2) |
| | | | No-postbiotic | 5/28 (17.9) |
| | | Southwest | Postbiotic | 0/0 (0) |
| | | | No-postbiotic | 0/0 (0) |
| Overall/feed additive status | | | Postbiotic | 100/847 (11.8) |
| | | | No-postbiotic | 291/1,969 (14.8) |
| Overall/region | | | Northeast | 110/1,432 (7.7) |
| | | | Southwest | 281/1,384 (20.3) |
| Overall/season | | | Summer | 84/791 (10.6) |
| | | | Fall | 84/656 (12.8) |
| | | | Winter | 131/652 (20.0) |
| | | | Spring | 92/717 (12.8) |
| Total | | | | 391/2,816 (13.9) |

Proportion positive = number of positive LN samples in each sampling/number of LN samples tested in each sampling.

Summer (June, July, August), fall (September, October, November), winter (December, January, February), spring (March, April, May).

Table 2.2. Study 1: Proportion and percentage of *Salmonella* test-positive lymph nodes of cull dairy cattle by season (and year), month, region, and feed additive status.

| Serotype | Northeast | | Southwest | | Total by serotype (%) |
|---|---|---|---|---|---|
| | Postbiotic (%) | No-postbiotic (%) | Postbiotic (%) | No-postbiotic (%) | |
| Montevideo | 17/60 (28.3) | 18/50 (36.0) | 30/140 (21.4) | 41/141 (29.1) | 106/391 (27.1) |
| Mbandaka | 7/60 (11.7) | 7/50 (14.0) | 23/140 (16.4) | 27/141 (19.1) | 64/391 (16.4) |
| Muenster | 2/60 (3.3) | - | 35/140 (25.0) | 17/141 (12.1) | 55/391 (14.1) |
| Cerro | 24/60 (40.0) | 13/50 (26.0) | 3/140 (2.1) | 2/141 (1.4) | 41/391 (10.5) |
| Meleagridis | - | - | 8/140 (5.7) | 14/141 (9.9) | 22/391 (5.6) |
| Anatum | 3/60 (5.0) | 1/50 (2.0) | - | 11/141 (7.8) | 19/391 (4.9) |
| Kentucky | 4/60 (6.7) | 4/50 (8.0) | - | 2/141 (1.4) | 10/391 (2.6) |
| Muenchen | - | - | 4/140 (2.9) | 4/141 (2.8) | 8/391 (2.0) |
| Bere | - | 1/50 (2.0) | 1/140 (0.7) | 4/141 (2.8) | 6/391 (1.5) |
| Give | - | - | 1/140 (0.7) | 4/141 (2.8) | 5/391 (1.3) |
| Rough_O:gms:- | - | 2/50 (4.0) | 3/140 (2.1) | - | 5/391 (1.3) |
| Fresno | - | - | 2/140 (1.4) | 3/141 (2.1) | 5/391 (1.3) |
| Typhimurium | 2/60 (3.3) | 1/50 (2.0) | - | 2/141 (1.4) | 5/391 (1.3) |
| Sundsvall | - | - | 4/140 (2.9) | - | 4/391 (1.0) |
| Bergen | - | - | 3/140 (2.1) | - | 3/391 (0.8) |
| Havana | - | - | 2/140 (1.4) | 1/141 (0.7) | 3/391 (0.8) |
| Oranienburg | | | 3/140 (2.1) | | 3/391 (0.8) |
| Brandenburg | 1/60 (1.7) | - | - | 1/141 (0.7) | 2/391 (0.5) |
| Kiambu | - | - | - | 2/141 (1.4) | 2/391 (0.5) |
| Newport | - | - | 2/140 (1.4) | - | 2/391 (0.5) |
| Rough_O:z:1,6 | - | - | - | 2/141 (1.4) | 2/391 (0.5) |
| Rough_O:e,h:1,6 | | | 2/140 (1.4) | | 2/391 (0.5) |
| Agona | - | - | 1/140 (0.7) | - | 1/391 (0.3) |
| Albany | - | - | - | 1/141 (0.7) | 1/391 (0.3) |
| Altona | - | - | 1/140 (0.7) | - | 1/391 (0.3) |
| Gaminara | - | - | - | 1/141 (0.7) | 1/391 (0.3) |
| III Rough O:z4,z23:- | - | - | 1/140 (0.7) | - | 1/391 (0.3) |

| | | | | | |
|---|---|---|---|---|---|
| Kedougou_ | - | - | 1/140 (0.7) | - | 1/391 (0.3) |
| Rough_O: z38: - | - | - | 1/140 (0.7) | - | 1/391 (0.3) |
| Rough_O:eh:1,5 | - | - | 1/140 (0.7) | - | 1/391 (0.3) |
| Rough_O:fg:- | - | - | 1/140 (0.7) | - | 1/391 (0.3) |
| RoughO:i:l,w | - | - | 1/140 (0.7) | - | 1/391 (0.3) |
| Saintpaul | - | - | - | 1/141 (0.7) | 1/391 (0.3) |
| Sandiego | - | - | - | 1/141 (0.7) | 1/391 (0.3) |
| Senftenberg | - | 1/50 (2.0) | - | - | 1/391 (0.3) |
| Uganda | - | - | 1/140 (0.7) | - | 1/391 (0.3) |
| III_35:z4,z32:- | | | 1/140 (0.7) | | 1/391 (0.3) |
| 3,10:e,h:- | - | - | 1/140 (0.7) | - | 1/391 (0.3) |
| 6,7:g,m,s:e,n,z15 | | 1/50 (2.0) | - | - | 1/391 (0.3) |

Table 2.3. Study 1: Proportion and percentage of *Salmonella* antimicrobial resistant isolates in lymph nodes of cull dairy cattle by antimicrobial, season, region, and feed additive status.

| Season | Region | Feed additive status | Proportion of resistant isolates (%) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | **AMX & CLA** | **AMP** | **AZI** | **FOX** | **XLN** | **AXO** | **MERO** | **NAL** | **STR** | **CIP** | **TET** | **CHL** | **TMP & SMX** |
| Summer | NE | Postbiotic | 0/14 (0) | 0/14 (0) | 0/14 (0) | 1/14 (7.14) | 1/14 (7.14) | 0/14 (0) | 1/14 (7.14) | 0/14 (0) | 3/14 (21.43) | 0/14 (0) | 1/14 (7.14) | 0/14 (0) | 0/14 (0) |
| | | No-postbiotic | 0/31 (0) | 0/31 (0) | 0/31 (0) | 0/31 (0) | 1/31 (3.23) | 0/31 (0) | 0/31 (0) | 0/31 (0) | 5/31 (16.13) | 0/31 (0) | 0/31 (0) | 1/31 (3.23) | 0/31 (0) |
| | SW | Postbiotic | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) |
| | | No-postbiotic | 0/39 (0) | 0/39 (0) | 2/39 (5.13) | 1/39 (2.56) | 1/39 (2.56) | 0/39 (0) | 0/39 (0) | 0/39 (0) | 4/39 (10.26) | 1/39 (2.56) | 3/39 (7.69) | 3/39 (7.69) | 3/39 (7.69) |
| Fall | NE | Postbiotic | 0/11 (0) | 0/11 (0) | 0/11 (0) | 0/11 (0) | 0/11 (0) | 0/11 (0) | 0/11 (0) | 0/11 (0) | 2/11 (18.18) | 0/11 (0) | 0/11 (0) | 0/11 (0) | 0/11 (0) |
| | | No-postbiotic | 0/3 (0) | 0/3 (0) | 0/3 (0) | 0/3 (0) | 0/3 (0) | 0/3 (0) | 0/3 (0) | 0/3 (0) | 0/3 (0) | 1/3 (33.33) | 0/3 (0) | 0/3 (0) | 0/3 (0) |
| | SW | Postbiotic | 0/27 (0) | 0/27 (0) | 0/27 (0) | 0/27 (0) | 0/27 (0) | 0/27 (0) | 0/27 (0) | 0/27 (0) | 3/27 (11.11) | 0/27 (0) | 1/27 (3.70) | 0/27 (0) | 0/27 (0) |
| | | No-postbiotic | 0/43 (0) | 0/43 (0) | 0/43 (0) | 0/43 (0) | 0/43 (0) | 0/43 (0) | 0/43 (0) | 0/43 (0) | 5/43 (11.63) | 2/43 (4.65) | 0/43 (0) | 0/43 (0) | 0/43 (0) |
| Winter | NE | Postbiotic | 0/9 (0) | 0/9 (0) | 0/9 (0) | 0/9 (0) | 0/9 (0) | 0/9 (0) | 0/9 (0) | 0/9 (0) | 2/9 (22.22) | 0/9 (0) | 2/9 (0) | 0/9 (0) | 0/9 (0) |
| | | No-postbiotic | 0/14 (0) | 0/14 (0) | 0/14 (0) | 0/14 (0) | 0/14 (0) | 0/14 (0) | 0/14 (0) | 0/14 (0) | 0/14 (0) | 1/14 (7.14) | 0/14 (0) | 0/14 (0) | 0/14 (0) |
| | SW | Postbiotic | 0/22 (0) | 0/22 (0) | 0/22 (0) | 0/22 (0) | 0/22 (0) | 0/22 (0) | 0/22 (0) | 0/22 (0) | 0/22 (0) | 1/22 (4.55) | 0/22 (0) | 0/22 (0) | 0/22 (0) |
| | | No-postbiotic | 0/86 (0) | 0/86 (0) | 0/86 (0) | 2/86 (2.33) | 0/86 (0) | 0/86 (0) | 0/86 (0) | 0/86 (0) | 5/86 (5.81) | 6/86 (6.98) | 0/86 (0) | 0/86 (0) | 0/86 (0) |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Spring | NE | Postbiotic | 0/9 (0) | 0/9 (0) | 0/9 (0) | 0/9 (0) | 1/9 (11.11) | 0/9 (0) | 0/9 (0) | 0/9 (0) | 1/9 (11.11) | 1/9 (11.11) | 1/9 (11.11) | 0/9 (0) | 0/9 (0) |
| | | No-postbiotic | 1/19 (5.26) | 1/19 (5.26) | 0/19 (0) | 1/19 (5.26) | 1/19 (5.26) | 1/19 (5.26) | 0/19 (0) | 1/19 (5.26) | 2/19 (10.53) | 12/19 (63.16) | 1/19 (5.26) | 1/19 (5.26) | 0/19 (0) |
| | SW | Postbiotic | 0/8 (0) | 0/8 (0) | 0/8 (0) | 0/8 (0) | 0/8 (0) | 0/8 (0) | 0/8 (0) | 0/8 (0) | 0/8 (0) | 1/8 (12.5) | 0/8 (0) | 0/8 (0) | 0/8 (0) |
| | | No-postbiotic | 0/56 (0) | 1/56 (1.79) | 0/56 (0) | 0/56 (0) | 0/56 (0) | 0/56 (0) | 0/56 (0) | 0/56 (0) | 2/56 (3.57) | 0/56 (0) | 3/56 (5.36) | 1/56 (1.79) | 0/56 (0) |
| Total/feed additive status | | Postbiotic | 0/100 (0) | 0/100 (0) | 0/100 (0) | 1/100 (1.00) | 1/100 (1.00) | 0/100 (0) | 1/100 (1.00) | 0/100 (0) | 11/100 (11.00) | 3/100 (3.00) | 3/100 (3.00) | 0/100 (0) | 0/100 (0) |
| | | No-postbiotic | 1/291 (0.34) | 2/291 (0.69) | 2/291 (0.69) | 4/291 (1.37) | 3/291 (1.03) | 1/291 (0.34) | 0/291 (0) | 1/291 (0.34) | 23/291 (7.90) | 23/291 (7.90) | 7/291 (2.41) | 6/291 (2.06) | 3/291 (1.03) |
| Total/region | | NE | 1/110 (0.92) | 1/110 (0.92) | 0/110 | 2/110 (1.82) | 3/110 (2.73) | 1/110 (0.92) | 1/110 (0.92) | 1/110 (0.92) | 15/110 (13.64) | 15/110 (13.64) | 3/110 (2.73) | 2/110 (1.82) | 0/110 |
| | | SW | 0/281 (0) | 1/281 (0.36) | 2/281 (0.71) | 3/281 (1.07) | 1/281 (0.36) | 0/281 (0) | 0/281 (0) | 0/281 (0) | 19/281 (6.76) | 11/281 (3.91) | 7/281 (2.49) | 4/281 (1.42) | 3/281 (1.07) |
| Total/season | | Summer | 0/84 (0) | 0/84 (0) | 2/84 (2.38) | 2/84 (2.38) | 2/84 (2.38) | 0/84 (0) | 1/84 (1.19) | 0/84 (0) | 12/84 (14.29) | 1/84 (1.19) | 4/84 (4.76) | 4/84 (4.76) | 3/84 (3.57) |
| | | Fall | 0/84 (0) | 0/84 (0) | 0/84 (0) | 0/84 (0) | 0/84 (0) | 0/84 (0) | 0/84 (0) | 0/84 (0) | 10/84 (11.90) | 3/84 (3.57) | 1/84 (1.19) | 0/84 (0) | 0/84 (0) |
| | | Winter | 0/131 (0) | 0/131 (0) | 0/131 (0) | 2/131 (1.53) | 0/131 (0) | 0/131(0) | 0/131 (0) | 0/131 (0) | 7/131 (5.34) | 8/131 (6.10) | 0/131 (0) | 0/131 (0) | 0/131 (0) |
| | | Spring | 1/92 (1.09) | 2/92 (2.17) | 0/92 (0) | 1/92 (1.09) | 2/92 (2.17) | 1/92 (1.09) | 0/92 (0) | 1/92 (1.09) | 5/92 (5.44) | 14/92 (15.22) | 5/92 (5.44) | 2/92 (2.17) | 0/92 (0) |
| Total | | | 1/391 (0.26) | 2/391 (0.51) | 2/391 (0.51) | 5/391 (1.28) | 4/391 (1.02) | 1/391 (0.26) | 1/391 (0.26) | 1/391 (0.26) | 34/391 (8.70) | 26/391 (6.65) | 10/391 (2.56) | 6/391 (1.53) | 3/391 (0.77) |

Proportion of resistant isolates = number of resistant isolates to a specific antimicrobial from LN samples per sampling / number of isolates from LN samples tested in each sampling.

Summer (June, July, August), fall (September, October, November), winter (December, January, February), spring (March, April, May).

Antimicrobial acronyms: AMX & CLA = Amoxicillin and Clavulanic acid combined; AMP = Ampicillin; FOX = Cefoxitin; XLN = Ceftiofur; AXO = Ceftriaxone; CHL = Chloramphenicol; CIP = Ciprofloxacin; NAL = Nalidixic acid; STR = Streptomycin; TET = Tetracycline; AZI = Azithromycin; MERO = Meropenem; TMP & SMX combination of Trimethoprim and Sulfamethoxazole. Gentamicin was also tested, but no resistant isolates were identified.

Table 2.4. Study 1: Number and resistance phenotypes of multi-drug resistant *Salmonella* isolates by season (and year), region and feed additive status.

| Season (Year) | Region | Feed additive status | Serotype (# of isolates) | Resistance phenotypes |
|---|---|---|---|---|
| Spring (2021) | Northeast | Postbiotic | Kentucky (1) | XLN, STR, TET |
| Summer (2021) | Southwest | No-postbiotic | Newport (2) | AZI, CHL, TET, SXT (TMP/SMX) |
| Summer (2021) | Southwest | No-postbiotic | Montevideo (1) | XLN, TET, SXT (TMP/SMX) |
| Spring (2023) | Northeast | No-postbiotic | Typhimurium (1) | AUG (AMX/CLA), AMP, FOX, XLN, AXO, CHL, CIP, NAL, STR, TET |
| Spring (2023) | Southwest | No-postbiotic | Anatum (1) | AMP, CHL, STR, TET |
| Summer (2023) | Southwest | No-postbiotic | Oranienburg (1) | FOX, CHL, CIP |

Antimicrobial acronyms: AMX & CLA = Amoxicillin and Clavulanic acid combined; AMP = Ampicillin; FOX = Cefoxitin; XLN = Ceftiofur; AXO = Ceftriaxone; CHL = Chloramphenicol; CIP = Ciprofloxacin; NAL = Nalidixic acid; STR = Streptomycin; TET = Tetracycline; AZI = Azithromycin; TMP & SMX combination of Trimethoprim and Sulfamethoxazole.

Meropenem was also tested but was not observed among the multi-drug resistance phenotypes for the identified *Salmonella* serotypes.

Antimicrobial classes: B-lactams (AUG, AMP, FOX, XLN, AXO, and MERO), Macrolides (AZI), Phenicols (CHL), Quinolones (CIP and NAL), Aminoglycosides (GEN and STR), Sulfonamides (FIS and SMX), Tetracyclines (TET), and Diaminopyrimidines (TMP).

Table 2.5. Study 1: Mean within-supplier *Salmonella* lymph node (LN) prevalence by feed additive status, season and region, from univariable models[1].

| Variable | ***Mean* within-supplier *Salmonella* LN prevalence, %** | **SEM, %** | **Mean within-supplier *Salmonella* LN prevalence, 95% CI, %** | ***P*-value** |
|---|---|---|---|---|
| **Feed additive status** | | | | 0.498 |
| Postbiotic | 11.80 | 5.76 | 0.07 – 96.30 | |
| No-postbiotic | 13.20 | 6.18 | 0.02 – 98.98 | |
| **Season** | | | | 0.308 |
| Summer | 11.79 | 5.74 | 0.16 – 91.61 | |
| Fall | 12.12 | 5.90 | 0.18 – 91.13 | |
| Winter | 16.53 | 7.52 | 0.16 – 96.07 | |
| Spring | 11.14 | 5.47 | 0.15 – 91.28 | |
| **Region** | | | | <0.001 |
| Northeast | 7.80 | 1.29 | 5.59 – 10.77 | |
| Southwest | 20.01 | 1.92 | 16.50 – 24.06 | |

[1] Univariable models assessed one variable at a time (feed additive status, season or region) with the mean within-supplier prevalence of *Salmonella* in LN. Models included a first-order autoregressive covariance structure for month of sampling within year, to account for repeated measures.

SEM = standard error of the mean; CI = confidence interval.

Table 2.6. Study 1: Model-adjusted mean within-supplier *Salmonella* lymph node (LN) prevalence based on feed additive status, season and region, from a multivariable model[1].

| Variable | Mean within-supplier *Salmonella* LN prevalence, % | SEM, % | Mean within-supplier *Salmonella* LN prevalence 95% CI, % | *P*-value |
|---|---|---|---|---|
| **Feed additive status** | | | | 0.485 |
| Postbiotic | 11.65 | 1.81 | 8.53 – 15.70 | |
| No-postbiotic | 13.10 | 1.38 | 10.60 – 16.07 | |
| **Season** | | | | 0.319 |
| Summer | 11.51 | 2.19 | 7.83 – 16.61 | |
| Fall | 11.88 | 2.27 | 8.06 – 17.16 | |
| Winter | 15.93 | 2.62 | 11.41 – 21.82 | |
| Spring | 10.62 | 2.08 | 7.15 – 15.49 | |
| **Region** | | | | <0.001 |
| Northeast | 7.76 | 1.30 | 5.55 – 10.75 | |
| Southwest | 19.10 | 2.03 | 15.40 – 23.43 | |

[1]Estimates from a multivariable model that included fixed effects for feed additive status, season and region, and a first-order autoregressive covariance structure for month of sampling within year, to account for repeated measures.

SEM = standard error of the mean; CI = confidence interval.

Table 2.7. Study 1: Unconditional [1] associations between feed additive status, season, or region with the probability of isolating a *Salmonella* serotype (Montevideo, Muenster, or Mbandaka compared to other serotypes (referent)).

| Variable | *Salmonella* serotype probability, % | | | | | | *P*-value |
|---|---|---|---|---|---|---|---|
| | Montevideo vs. Other serotypes | | Muenster vs. Other serotypes | | Mbandaka vs. Other serotypes | | |
| | Mean probability, % | SEM, % | Mean probability, % | SEM, % | Mean probability, % | SEM, % | |
| **Feed additive status** | | | | | | | 0.434 |
| Postbiotic | 11.15 | 7.33 | 6.61 | 4.94 | 12.72 | 8.38 | |
| No-postbiotic | 26.98 | 6.95 | 10.20 | 3.98 | 13.31 | 4.84 | |
| **Season** | | | | | | | 0.001 |
| Summer | 28.38 | 8.19 | 3.50 | 2.25 | 8.04 | 3.94 | |
| Fall | 31.80 | 9.70 | 11.26 | 5.00 | 7.85 | 4.05 | |
| Winter | 11.00 | 4.29 | 18.13 | 6.30 | 15.84 | 5.77 | |
| Spring | 23.03 | 7.53 | 6.02 | 3.07 | 20.72 | 7.48 | |
| **Region** | | | | | | | 0.097 |
| Northeast | 23.20 | 7.75 | 3.32 | 2.20 | 11.03 | 4.99 | |
| Southwest | 19.19 | 8.53 | 19.09 | 6.66 | 15.31 | 7.32 | |

[1]Based on univariable models that included fixed effects for feed additive status, season, or region (i.e., testing one fixed effect at a time) and a random intercept for supplier

Summer (June, July, August), fall (September, October, November), winter (December, January, February), spring (March, April, May).

SEM = standard error of the mean; CI = confidence interval.

Table 2.8. Study 1: Final multivariable model[1] of the association between feed additive status, season, and region on the probability of isolating a *Salmonella* serotype (Montevideo, Muenster, Mbandaka or other serotypes (referent)).

| Variable | *Salmonella* serotype probability, % | | | | | | *P*-value |
|---|---|---|---|---|---|---|---|
| | Montevideo vs. Other serotypes | | Muenster vs. Other serotypes | | Mbandaka vs. Other serotypes | | |
| | Mean probability, % | SEM, % | Mean probability, % | SEM, % | Mean probability, % | SEM, % | |
| **Feed additive status** | | | | | | | 0.385 |
| Postbiotic | 10.25 | 6.95 | 5.65 | 4.36 | 11.04 | 7.88 | |
| No-postbiotic | 29.20 | 7.89 | 8.02 | 3.69 | 12.57 | 5.07 | |
| **Season[2]** | | | | | | | 0.002 |
| Summer | 21.73 | 8.25 | 3.26 | 2.33 | 7.98 | 4.57 | |
| Fall | 26.51 | 9.53 | 9.16 | 4.77 | 7.88 | 4.44 | |
| Winter | 8.38 | 3.88 | 13.97 | 6.21 | 14.99 | 6.56 | |
| Spring | 18.45 | 7.24 | 4.89 | 2.83 | 20.32 | 8.39 | |
| **Region** | | | | | | | 0.362 |
| Northeast | 17.50 | 7.38 | 3.51 | 2.43 | 10.60 | 5.70 | |
| Southwest | 17.68 | 8.37 | 13.34 | 6.60 | 13.54 | 7.26 | |

[1]Multivariable model included fixed effects for feed additive status, season and region, and a random intercept for supplier within region.

Summer (June, July, August), fall (September, October, November), winter (December, January, February), spring (March, April, May).

[2]Significant contrasts included Montevideo vs. Other serotypes - fall vs. winter ($P = 0.004$), Montevideo vs. Other serotypes - Spring vs winter ($P = 0.035$), and Muenster vs. Other serotypes – winter vs. Summer ($P = 0.028$).

SEM = standard error of the mean; CI = confidence interval.

Table 2.9. Study 1: Associations between feed additive status, season, and region with the probability of a *Salmonella* isolate being resistant to streptomycin or to ciprofloxacin, from univariable models[1].

| Variable | Streptomycin | | | Ciprofloxacin | | |
|---|---|---|---|---|---|---|
| | **Mean probability of *Salmonella* resistance, %** | **SEM, %** | ***P*-value** | **Mean probability of *Salmonella* resistance, %** | **SEM, %** | ***P*-value** |
| **Feed additive status** | | | 0.198 | | | 0.724 |
| Postbiotic | 1.63 | 1.37 | - | 9.96 | 4.61 | - |
| No-postbiotic | 4.68 | 2.43 | - | 8.66 | 3.51 | - |
| **Season** | | | 0.510 | | | 0.310 |
| Summer | 0.88 | 1.15 | - | 12.96 | 5.84 | - |
| Fall | 3.64 | 3.64 | - | 13.07 | 6.34 | - |
| Winter | 4.97 | 4.62 | - | 5.12 | 3.03 | - |
| Spring | 7.59 | 6.18 | - | 5.82 | 3.45 | - |
| **Region** | | | 0.264 | | | 0.481 |
| Northeast | 5.80 | 3.24 | - | 13.26 | 3.89 | - |
| Southwest | 2.57 | 1.57 | - | 6.29 | 1.83 | - |

[1]Univariable models were fitted by including one fixed effect at a time, and random intercepts for supplier, month within year, and region (when region was not included as fixed effect)). Models were run separately for each outcome (probability of *Salmonella* isolates resistant to streptomycin, and probability of *Salmonell*a isolates resistant to ciprofloxacin).

Summer (June, July, August), fall (September, October, November), winter (December, January, February), spring (March, April, May).

Table 2.10. Study 1: Answers to the questionnaire obtained from five dairy farm suppliers that supplied cattle for the study.

| Questions/Topics | Supplier #1 | Supplier #2 | Supplier #3 | Supplier #4 | Supplier #5 |
|---|---|---|---|---|---|
| **Farm Demographics** | | | | | |
| Heifers raised off-site, BIFSCo region[†] | - | Yes, region 6 | Yes, region 5 | Yes, region 5 | Yes, not specified |
| Number of cows on-site | >2,000 feedlot cows | >2,000 milking cows | 200 to 2,000 milking cows | 200 to 2,000 milking cows | >2,000 milking cows |
| Tie-stall housing; if no, specify | No; dry lots & open barns | No; free-stall | No; free-stall | No; free-stall | No; free-stall |
| **Biosecurity/Management Factors** | | | | | |
| Cattle bedding material, maintenance frequency | Corn stalks & bed pack, removed weekly | Biosolids, maintained daily | Sand, raked twice weekly | Sand, raked weekly | Sand, raked daily |
| Pre-weaned calves housed individually, paired, or grouped | Individually | Grouped (3 or more) | Individually | Individually | Individually |
| Perceived risk of *Salmonella* introduction via pests | No | Yes, birds | Yes, biting flies & birds | Yes, birds | Yes, birds |
| Current pest management strategies | No | Yes, wire mesh in open ridge & curtain sidewalls | Yes, spray cattle as needed & feed additives in rations for flies | Yes, fly spray weekly in summer, pigeon shooting, poisoning, starling poison by DNR | No |
| Methods to decrease heat stress | Sprinklers, access to shade, & fans | Sprinklers & fans | Sprinklers, access to shade, & fans | Sprinklers & fans | Sprinklers & fans |

| | | | | | |
|---|---|---|---|---|---|
| Current use of products for reduction/prevention of *Salmonellosis* | - | SRP vaccine | Endovac-Beef (*Salmonella* typhimurium and other gram-negative bacteria) | None | SRP vaccine |
| **Cull Cattle Information** | | | | | |
| Top 3 production reasons for culling cattle at the time of this questionnaire | - | Long open, low milk due to genetic merit, abortion | Production, breeding, health | Too many days in milk, abort, feet/legs | Reproduction, mastitis, feet |
| Top 3 disease reasons for culling cattle at the time of this questionnaire | - | Mastitis, SCC, lame | Respiratory, SCC | Mastitis, pneumonia, metritis | Mastitis, respiratory |
| Number of cull animals (in this shipment) represented by production or disease | [Production: 5,000 heifers; 8,000 cows; 5,000 bull/steers]<br>Disease: - | Production: 7 cows<br>Disease: 1 cow | Production: 13 cows<br>Disease: 0 | Production: 5 cows<br>Disease: 0 | - |
| For mature cows culled, number represented by each parity category | - | First parity: 1<br>Second parity: 1<br>Third parity: 1<br>Fourth or greater parity: 5 | First parity: 1<br>Second parity: 2<br>Third parity: 3<br>Fourth or greater parity: 7 | First parity: 1<br>Second parity: 1<br>Third parity: 2<br>Fourth or greater parity: 1 | - |
| Number of mature cows culled at what stage of production | - | Early lactation: 1<br>Mid-lactation: 1<br>Late lactation: 6<br>Dry period: 0 | Early lactation: 1<br>Mid-lactation: 2<br>Late lactation: 10<br>Dry period: - | Early lactation: 0<br>Mid-lactation: 1<br>Late lactation: 4<br>Dry period: 0 | - |
| Average days in milk (DIM) at each stage of production when culled | - | Early lactation: 35 DIM | Early lactation: 70 DIM, range of DIM=70 | Early lactation: 0 | - |

| | | | |
|---|---|---|---|
| | Mid-lactation: 150 DIM<br>Late Lactation: 300 DIM, range of DIM=200-650 | Mid-lactation: 120 DIM, range of DIM=100-146<br>Late Lactation: 320 DIM, range of DIM=175-400 | Mid-lactation: 256 DIM, range of DIM=256<br>Late Lactation: 498 DIM, range of DIM=352-843 |

Suppliers #1 through 5 belong to No-postbiotic farms.

[†]BIFSCo regions: Region 1: Washington, Oregon, Idaho; Region 2: California, Nevada; Region 3: Arizona, New Mexico, Texas; Region 4: Montana, Wyoming, Utah, Colorado; Region 5: North Dakota, South Dakota, Nebraska, Minnesota, Wisconsin; Region 6: Kansas, Iowa, Missouri; Region 7: Oklahoma, Arkansas, Louisiana, Mississippi, Tennessee, Alabama, Georgia, North Carolina, South Carolina, Florida; Region 8: Illinois, Michigan, Indiana, Kentucky, Ohio, West Virginia, Virginia, Maryland, Washington D.C., Delaware, Pennsylvania, New Jersey, Connecticut, Rhode Island, New York, Massachusetts, Vermont, New Hampshire, Maine.

Table 2.11. Study 2: Proportion and percentage of *Salmonella* test-positive lymph nodes of cull dairy cattle by season (and year), month, and region.

| Season (year) | Month | Region | Proportion positives[1] (%) |
|---|---|---|---|
| Fall (2022) | September | Upper Midwest | 0/14 (0) |
| | | Midwest | 0/0 (0) |
| | | Northeast | 0/0 (0) |
| | | Southwest | 4/16 (25.0) |
| | October | Upper Midwest | 8/56 (14.3) |
| | | Midwest | 0/0 (0) |
| | | Northeast | 0/40 (0) |
| | | Southwest | 6/11 (54.5) |
| | November | Upper Midwest | 3/56 (5.4) |
| | | Midwest | 0/0 (0) |
| | | Northeast | 1/23 (4.3) |
| | | Southwest | 6/19 (31.6) |
| Winter (2022-23) | December | Upper Midwest | 2/28 (7.1) |
| | | Midwest | 0/0 (0) |
| | | Northeast | 2/26 (7.7) |
| | | Southwest | 21/57 (36.8) |
| | January | Upper Midwest | 4/66 (6.1) |
| | | Midwest | 0/0 (0) |
| | | Northeast | 5/49 (10.2) |
| | | Southwest | 33/132 (25.0) |
| | February | Upper Midwest | 8/64 (12.5) |
| | | Midwest | 0/0 (0) |
| | | Northeast | 5/36 (13.9) |
| | | Southwest | 25/79 (31.6) |
| Spring (2023) | March | Upper Midwest | 10/96 (10.4) |
| | | Midwest | 0/0 (0) |
| | | Northeast | 0/0 (0) |
| | | Southwest | 17/78 (21.8) |
| | April | Upper Midwest | 9/87 (10.3) |

| | | | |
|---|---|---|---|
| | | Midwest | 8/40 (20.0) |
| | | Northeast | 2/46 (4.3) |
| | | Southwest | 13/80 (16.3) |
| | May | Upper Midwest | 8/98 (8.2) |
| | | Midwest | 6/80 (7.5) |
| | | Northeast | 7/103 (6.8) |
| | | Southwest | 17/79 (21.5) |
| Summer (2023) | June | Upper Midwest | 11/73 (15.1) |
| | | Midwest | 1/80 (1.3) |
| | | Northeast | 8/72 (11.1) |
| | | Southwest | 16/118 (13.6) |
| | July | Upper Midwest | 15/72 (20.8) |
| | | Midwest | 10/60 (16.7) |
| | | Northeast | 9/78 (11.5) |
| | | Southwest | 7/119 (5.9) |
| Overall/region | | Upper Midwest | 78/710 (11.0) |
| | | Midwest | 25/260 (9.6) |
| | | Northeast | 39/473 (8.2) |
| | | Southwest | 165/788 (20.9) |
| Overall/season | | Summer | 77/672 (11.5) |
| | | Fall | 28/235 (11.9) |
| | | Winter | 105/537 (19.6) |
| | | Spring | 97/787 (12.3) |
| Total | | | 307/2,231 (13.8) |

[1]Proportion positive = number of positive LN samples in each sampling/number of LN samples tested in each sampling.

Fall (September, October, November), winter (December, January, February), spring (March, April, May), summer (June, July, August).

Table 2.12. Study 2: Proportion and percentage of *Salmonella* serotypes isolated from the subiliac lymph nodes of cull dairy cattle across all seasons, by region.

| Serotype | Upper Midwest | Midwest | Northeast | Southwest | Total by serotype (%) |
|---|---|---|---|---|---|
| Montevideo | 19/78 (24.4) | - | 8/39 (20.5) | 32/165 (19.4) | 59/307 (19.2) |
| Muenster | 10/78 (12.8) | 7/25 (28.0) | 5/39 (12.8) | 31/165 (18.8) | 53/307 (17.3) |
| Mbandaka | 6/78 (8.0) | - | 4/39 (10.3) | 37/165 (22.4) | 47/307 (15.3) |
| Anatum | 14/78 (17.9) | 9/25 (36.0) | 2/39 (5.1) | 13/165 (7.9) | 38/307 (12.4) |
| Cerro | 18/78 (23.1) | - | 7/39 (17.9) | 4/165 (2.4) | 29/307 (9.4) |
| Meleagridis | - | - | - | 11/165 (6.7) | 11/307 (3.6) |
| Kentucky | 3/78 (3.8) | - | 6/39 (15.4) | - | 9/307 (2.9) |
| Typhimurium | - | 5/25 (20.0) | 2/39 (5.1) | 2/165 (1.2) | 9/307 (2.9) |
| Bere | 3/78 (3.8) | - | 1/39 (2.6) | 4/165 (2.4) | 8/307 (2.6) |
| Give | - | - | - | 5/165 (3.0) | 5/307 (1.6) |
| Sundsvall | - | - | 1/39 (2.6) | 4/165 (2.4) | 5/307 (1.6) |
| Muenchen | - | 1/25 (4.0) | - | 3/165 (1.8) | 4/307 (1.3) |
| Rough O:z:1,6 | 2/78 (2.6) | - | - | 2/165 (1.2) | 4/307 (1.3) |
| Altona | - | 2/25 (8.0) | - | 1/165 (0.6) | 3/307 (1.0) |
| Fresno | - | - | - | 3/165 (1.8) | 3/307 (1.0) |
| III_35:z4,z32:- | - | - | 2/39 (5.1) | 1/165 (0.6) | 3/307 (1.0) |
| Oranienburg | - | - | - | 3/165 (1.8) | 3/307 (1.0) |
| Rough_O:e,h:1,6 | 1/78 (1.3) | - | - | 2/165 (1.2) | 3/307 (1.0) |
| Anatum_var._15+ | 1/78 (1.3) | - | - | - | 1/307 (0.3) |
| Albany | - | - | - | 1/165 (0.6) | 1/307 (0.3) |
| Brandenburg | - | - | - | 1/165 (0.6) | 1/307 (0.3) |
| Dublin | 1/78 (1.3) | - | - | - | 1/307 (0.3) |
| Gaminara | - | - | - | 1/165 (0.6) | 1/307 (0.3) |
| Havana | - | - | - | 1/165 (0.6) | 1/307 (0.3) |
| III Rough O:z4,z23:- | - | - | - | 1/165 (0.6) | 1/307 (0.3) |
| Rough O:e,h:l,w | - | 1/25 (4.0) | - | - | 1/307 (0.3) |

| | | | | | |
|---|---|---|---|---|---|
| Rough_O:eh:1,5 | - | - | - | 1/165 (0.6) | 1/307 (0.3) |
| Saintpaul | - | - | - | 1/165 (0.6) | 1/307 (0.3) |
| 6,7:g,m,s:e,n,z15 | - | - | 1/39 (2.6) | - | 1/307 (0.3) |

Table 2.13. Study 2: Proportion and percentage of *Salmonella* isolates resistant to antimicrobials in lymph nodes of cull dairy cattle, by antimicrobial evaluated and by season, and region.

| Season | Region | Proportion of resistant isolates (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **AMX & CLA** | **AMP** | **FOX** | **XLN** | **AXO** | **NAL** | **STR** | **CIP** | **TET** | **CHL** |
| Summer | NE | 0/17 (0) | 1/17 | 0/17 (0) | 0/17 (0) | 0/17 (0) | 0/17 (0) | 4/17 (23.53) | 0/17 (0) | 0/17 (0) | 0/17 (0) |
| | SW | 0/23 (0) | 0/23 (0) | 1/23 (4.35) | 0/23 (0) | 0/23 (0) | 0/23 (0) | 2/23 (8.70) | 1/23 (4.35) | 0/23 (0) | 1/23 (4.35) |
| | MW | 0/11 (0) | 0/11 (0) | 0/11 (0) | 0/11 (0) | 0/11 (0) | 0/11 (0) | 1/11 (9.09) | 0/11 (0) | 1/11 (9.09) | 0/11 (0) |
| | UMW | 1/26 (3.85) | 1/26 (3.85) | 2/26 (7.69) | 1/26 (3.85) | 3/26 (11.54) | 0/26 (0) | 1/26 (3.85) | 1/26 (3.85) | 1/26 (3.85) | 1/26 (3.85) |
| Fall | NE | 0/1 (0) | 0/1 (0) | 0/1 (0) | 0/1 (0) | 0/1 (0) | 0/1 (0) | 0/1 (0) | 1/1 (100) | 0/1 (0) | 0/1 (0) |
| | SW | 0/16 (0) | 0/16 (0) | 0/16 (0) | 0/16 (0) | 0/16 (0) | 0/16 (0) | 3/16 (18.75) | 2/16 (12.50) | 0/16 (0) | 0/16 (0) |
| | MW | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) |
| | UMW | 0/11 (0) | 0/11 (0) | 0/11 (0) | 0/11 (0) | 0/11 (0) | 0/11 (0) | 0/11 (0) | 3/11 (27.27) | 0/11 (0) | 0/11 (0) |
| Winter | NE | 0/12 (0) | 0/12 (0) | 0/12 (0) | 0/12 (0) | 0/12 (0) | 0/12 (0) | 0/12 (0) | 1/12 (8.33) | 0/12 (0) | 0/12 (0) |
| | SW | 0/79 (0) | 0/79 (0) | 2/79 (2.53) | 0/79 (0) | 0/79 (0) | 0/79 (0) | 5/79 (6.33) | 6/79 (7.59) | 0/79 (0) | 0/79 (0) |
| | MW | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) | 0/0 (0) |
| | UMW | 0/14 (0) | 0/14 (0) | 0/14 (0) | 0/14 (0) | 0/14 (0) | 0/14 (0) | 2/14 (14.29) | 0/14 (0) | 0/14 (0) | 0/14 (0) |
| Spring | NE | 1/9 (11.11) | 1/9 (11.11) | 1/9 (11.11) | 1/9 (11.11) | 1/9 (11.11) | 1/9 (11.11) | 2/9 (22.22) | 1/9 (11.11) | 0/9 (0) | 1/9 (11.11) |
| | SW | 0/47 (0) | 1/47 (2.13) | 0/47 (0) | 0/47 (0) | 0/47 (0) | 0/47 (0) | 2/47 (4.26) | 0/47 (0) | 3/47 (6.38) | 1/47 (2.13) |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | MW | 0/14 (0) | 0/14 (0) | 0/14 (0) | 0/14 (0) | 0/14 (0) | 0/14 (0) | 5/14 (35.71) | 0/14 (0) | 1/14 (7.14) | 0/14 (0) |
| | UMW | 0/27 (0) | 1/27 (3.70) | 0/27 (0) | 0/27 (0) | 0/27 (0) | 0/27 (0) | 8/27 (29.63) | 0/27 (0) | 2/27 (7.41) | 1/27 (3.70) |
| Total/region | NE | 1/39 (2.56) | 1/39 (2.56) | 1/39 (2.56) | 1/39 (2.56) | 1/39 (2.56) | 1/39 (2.56) | 6/39 (15.38) | 3/39 (7.69) | 1/39 (2.56) | 1/39 (2.56) |
| | SW | 0/165 (0) | 1/165 (0.61) | 3/165 (1.81) | 0/165 (0) | 0/165 (0) | 0/165 (0) | 12/165 (7.27) | 9/165 (5.46) | 3/165 (1.81) | 2/165 (1.21) |
| | MW | 0/25 (0) | 0/25 (0) | 0/25 (0) | 0/25 (0) | 0/25 (0) | 0/25 (0) | 6/25 (24.0) | 0/25 (0) | 2/25 (8.00) | 0/25 (0) |
| | UMW | 1/78 (1.28) | 2/78 (2.56) | 2/78 (2.56) | 1/78 (1.28) | 3/78 (3.85) | 0/78 (0) | 11/78 (14.10) | 4/78 (5.13) | 3/78 (3.85) | 2/78 (2.56) |
| Total/season | Summer | 1/77 (1.30) | 1/77 (1.30) | 3/77 (3.90) | 1/77 (1.30) | 3/77 (3.90) | 0/77 (0) | 8/77 (10.39) | 2/77 (2.60) | 2/77 (2.60) | 2/77 (2.60) |
| | Fall | 0/28 (0) | 0/28 (0) | 0/28 (0) | 0/28 (0) | 0/28 (0) | 0/28 (0) | 3/28 (10.71) | 6/28 (21.43) | 0/28 (0) | 0/28 (0) |
| | Winter | 0/105 (0) | 0/105 (0) | 2/105 (1.91) | 0/105 (0) | 0/105 (0) | 0/105 (0) | 7/105 (6.67) | 7/105 (6.67) | 0/105 (0) | 0/105 (0) |
| | Spring | 1/97 (1.03) | 3/97 (3.09) | 1/97 (1.03) | 1/97 (1.03) | 1/97 (1.03) | 1/97 (1.03) | 17/97 (17.53) | 1/97 (1.03) | 7/97 (7.22) | 3/97 (3.09) |
| Total | | 2/307 (0.98) | 4/307 (1.30) | 6/307 (1.95) | 2/307 (0.98) | 4/307 (1.30) | 1/307 (0.33) | 35/307 (11.40) | 16/307 (5.21) | 9/307 (2.93) | 5/307 (1.63) |

Proportion of resistant isolates = number of resistant isolates from LN samples in each sampling/ susceptible isolates from LN samples tested in each sampling.

Summer (June, July, August), fall (September, October, November), winter (December, January, February), spring (March, April, May).

Antimicrobial acronyms: AMX & CLA = Amoxicillin and Clavulanic acid combined; AMP = Ampicillin; FOX = Cefoxitin; XLN = Ceftiofur; AXO = Ceftriaxone; CHL = Chloramphenicol; CIP = Ciprofloxacin; NAL = Nalidixic acid; STR = Streptomycin; TET = Tetracycline.

Gentamicin, Azithromycin, Meropenem, and the combination of Trimethoprim and Sulfamethoxazole were also tested, and no resistant isolates were identified.

NE = Northeast, SW = Southwest, MW = Midwest; UMW = Upper Midwest.

Table 2.14. Study 2: Number and resistance phenotypes of multi-drug resistant *Salmonella* isolates by season (and year), and region.

| Season (Year) | Region | Serotype (# of isolates) | Resistance phenotypes |
|---|---|---|---|
| Spring (2023) | Northeast | Typhimurium (1) | AUG (AMX/CLA), AMP, FOX, XLN, AXO, CHL, CIP, NAL, STR, TET |
| Spring (2023) | Southwest | Anatum (1) | AMP, CHL, STR, TET |
| Summer (2023) | Upper Midwest | Dublin (1) | TET, STR, CHL, AXO, XLN, FOX, AMP, AUG (AMX/CLA) |
| Summer (2023) | Southwest | Oranienburg (1) | FOX, CHL, CIP |

Antimicrobial acronyms: AMX & CLA = Amoxicillin and Clavulanic acid combined; AMP = Ampicillin; FOX = Cefoxitin; XLN = Ceftiofur; AXO = Ceftriaxone; CHL = Chloramphenicol; CIP = Ciprofloxacin; NAL = Nalidixic acid; STR = Streptomycin; TET = Tetracycline.

Meropenem, Azithromycin, and Trimethoprim/Sulfamethoxazole were also tested but were not observed among the multi-drug resistance phenotypes for the identified *Salmonella* serotypes.

Antimicrobial classes: B-lactams (AUG, AMP, FOX, XLN, AXO, and MERO), Macrolides (AZI), Phenicols (CHL), Quinolones (CIP and NAL), Aminoglycosides (GEN and STR), Sulfonamides (FIS and SMX), Tetracyclines (TET), and Diaminopyrimidines (TMP).

Table 2.15. Study 2: Model-adjusted mean within-supplier *Salmonella* lymph node prevalence by season and region from univariable models[1].

| Variable | ***Mean* within-supplier *Salmonella* LN prevalence, %** | **SEM, %** | **Mean within-supplier *Salmonella* LN prevalence, 95% CI, %** | ***P*-value** |
|---|---|---|---|---|
| **Season** | | | | 0.217 |
| Summer | 10.41 | 2.91 | 5.26 – 19.55 | |
| Fall | 12.10 | 4.35 | 5.52 – 24.50 | |
| Winter | 16.67 | 4.29 | 8.62 – 29.79 | |
| Spring | 11.32 | 2.99 | 5.77 – 21.04 | |
| **Region**† | | | | <0.001 |
| Upper Midwest | 11.46 | 2.01 | 8.05 – 16.07 | |
| Midwest | 9.92 | 3.10 | 5.27 – 17.91 | |
| Northeast | 7.59 | 2.10 | 4.35 – 12.94 | |
| Southwest | 21.30 | 2.44 | 16.87 – 26.52 | |

[1]Univariable models fitted one variable at a time (season or region) with the mean within-supplier prevalence of *Salmonella* in LN and included a first-order autoregressive covariance structure for month of sampling within year, to account for repeated measures.

†Significant contrasts include Southwest vs. Northeast ($P = 0.002$), and Southwest vs. Upper Midwest ($P = 0.016$).

SEM = standard error of the mean; CI = confidence interval

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Table 2.16. Study 2: Model-adjusted mean within-supplier *Salmonella* lymph node prevalence by season and region from a multivariable model[1].

| Variable | ***Mean* within-supplier *Salmonella* LN prevalence, %** | **SEM, %** | **Mean within-supplier *Salmonella* LN prevalence, 95% CI, %** | ***P*-value** |
|---|---|---|---|---|
| **Season** | | | | 0.679 |
| Summer | 11.44 | 2.06 | 7.93 – 16.23 | |
| Fall | Non-est. | - | - | |
| Winter | Non-est. | - | - | |
| Spring | 10.70 | 2.01 | 7.30 – 15.43 | |
| **Region** | | | | <0.001 |
| Upper Midwest | 10.85 | 1.96 | 7.53 – 15.39 | |
| Midwest | Non-est. | - | - | |
| Northeast | 5.83 | 2.39 | 2.56 – 12.75 | |
| Southwest | 21.97 | 2.83 | 16.89 – 28.06 | |
| **Season*region**[†] | - | - | - | 0.027 |
| Fall*NE | 1.64 | 2.44 | 0.08 – 24.86 | |
| Fall*SW | 34.78 | 10.51 | 17.63 – 57.07 | |
| Fall*UMW | 8.55 | 3.96 | 3.30 – 20.40 | |
| Fall*MW | - | - | - | |
| Spring*NE | 5.73 | 3.05 | 1.94 – 15.74 | |
| Spring*SW | 19.93 | 4.04 | 13.11 – 29.10 | |
| Spring*UMW | 9.80 | 2.75 | 5.55 – 16.74 | |
| Spring*MW | 11.16 | 4.47 | 4.90 – 23.43 | |
| Summer*NE | 10.75 | 4.03 | 4.96 – 21.74 | |
| Summer*SW | 10.10 | 3.08 | 5.43 – 18.00 | |
| Summer*UMW | 18.05 | 4.80 | 10.41 – 29.46 | |
| Summer*MW | 8.55 | 3.69 | 3.55 – 19.17 | |
| Winter*NE | 10.77 | 4.65 | 4.42 – 23.97 | |
| Winter*SW | 29.64 | 4.32 | 21.86 – 38.81 | |
| Winter*UMW | 8.94 | 3.62 | 3.88 – 19.24 | |
| Winter*MW | - | - | - | |

[1]Estimates from a multivariable model that included fixed effects for season, region, and the two-way interaction term between season and region, and a first-order autoregressive covariance structure for month of sampling within year, to account for repeated measures.

[†]Significant contrast included Spring*UMW vs. Winter*SW ($P = 0.026$).

Non-est. = non-estimable; SEM = standard error of the mean; CI = confidence interval.

Table 2.17. Study 2: Unconditional [1] associations between season with the probability of isolating a *Salmonella* serotype (Montevideo, Muenster, Mbandaka, and other serotypes (referent)).

| Variable | *Salmonella* serotype probability, % | | | | | | *P*-value |
|---|---|---|---|---|---|---|---|
| | Montevideo vs. Other serotypes | | Muenster vs. Other serotypes | | Mbandaka vs. Other serotypes | | |
| | Mean probability, % | SEM, % | Mean probability, % | SEM, % | Mean probability, % | SEM, % | |
| **Season** | | | | | | | 0.008 |
| Summer | 10.82 | 4.24 | 21.72 | 6.895 | 29.63 | 2.96 | |
| Fall | 36.40 | 13.17 | 20.63 | 11.84 | 8.16 | 5.54 | |
| Winter | 9.99 | 3.73 | 16.08 | 6.132 | 4.79 | 4.79 | |
| Spring | 20.31 | 6.07 | 4.00 | 2.254 | 8.44 | 4.11 | |

[1]Based on a univariable model that included season as a fixed effect and a random intercept for supplier nested within region.

Summer (June, July, August), fall (September, October, November), winter (December, January, February), spring (March, April, May).

Significant contrasts included Montevideo vs. Other serotypes - Fall vs. summer ($P = 0.011$), Montevideo vs. Other serotypes - Fall vs winter ($P = 0.004$), Muenster vs. Other serotypes - Fall vs spring ($P = 0.009$), Muenster vs. Other serotypes - Summer vs. spring ($P = 0.004$), and Muenster vs. Other serotypes - Winter vs. spring ($P = 0.015$).

SEM = standard error of the mean; CI = confidence interval.

Table[1] 2.18. Study 2: Associations between season, and region with the probability of a *Salmonella* isolate being resistant to streptomycin from univariable models[1].

| Variable | Streptomycin | | |
|---|---|---|---|
| | **Mean probability of *Salmonella* resistance, %** | **SEM, %** | ***P*-value** |
| **Season** | | | 0.762 |
| Summer | 13.26 | 7.03 | - |
| Fall | 9.58 | 7.07 | - |
| Winter | 6.84 | 3.37 | - |
| Spring | 12.69 | 5.58 | - |
| **Region** | | | 0.568 |
| Upper-Midwest | 13.81 | 5.68 | |
| Midwest | 17.86 | 9.98 | - |
| Northeast | 13.42 | 6.34 | - |
| Southwest | 7.56 | 2.72 | - |

[1]Univariable models were fitted by including one fixed effect at a time, and random intercepts for supplier, month within year, and region (when region was not included as fixed effect)).

Summer (June, July, August), fall (September, October, November), winter (December, January, February), spring (March, April, May).

# Chapter 3 - General discussion and conclusions

## 3.1 General discussion

Interest in pre-harvest intervention strategies aimed at reducing *Salmonella*-related human illness, linked to ground beef, has been the center of food safety research in recent times despite decades of advancements in this area. The general goal of this thesis was to add to the growing body of knowledge on research into food safety and pre-harvest intervention strategies that are being developed and implemented within the beef industry with the aim of reducing the incidence of *Salmonella* in cattle and to identify areas amenable for future research. Chapter 1 consists of a review of the existing literature on pre-harvest and post-harvest intervention strategies to reduce the burden of *Salmonella* in different cattle and environmental matrices, including feces, hides, feed, water, and the peripheral LN. Although previous studies have repeatedly implicated cattle hides and feces as the major sources of recovering *Salmonella* in cattle, the LN remains an important cattle matrix that has constantly received attention in recent research because of its uniqueness in protecting *Salmonella* organisms against post-harvest intervention strategies. Upon detailed assessment of the literature, we identified several risk factors including season, region, and cattle production system among others that are associated with the prevalence and concentration of *Salmonella* in cattle LN. Simultaneously, we also identified knowledge gaps, particularly pertaining to the practical implementation of pre-harvest interventions aimed at reducing *Salmonella* burden in real-world settings, which sets the stage for continued research.

Through an observational study (Chapter 2), we tested the effectiveness of a larger scale or farm-level feed supplementation of a postbiotic product in reducing *Salmonella* prevalence in the subiliac LN of cull dairy cattle at slaughter. Postbiotic products act by excluding harmful

bacteria and preventing their colonization in the digestive tract. Studies have shown the use of this postbiotic product to be efficacious at mitigating *Salmonella* in experimental studies. However, given the limited external validity of experimental studies, our goal was to evaluate the effectiveness of a postbiotic product in *Salmonella* LN prevalence reduction in real-world scenarios and via observational studies by collaborating with large commercial processing plants that receive cull dairy cattle from farms where this postbiotic product was administered. While this study did not demonstrate this postbiotic product as an effective intervention in reducing *Salmonella* LN prevalence in cull dairy, it did highlight the inherent difficulties in carrying out this type of research, which is consistent with previous studies in this field. The precise reasons for the observed ineffectiveness of the postbiotic product in this research remain uncertain. Nonetheless, several potential factors may have contributed to this outcome. For instance, the study included certain limitations, such as the enrollment of fewer postbiotic suppliers compared to No-postbiotic suppliers, and the lower than anticipated *Salmonella* prevalence found in LN of cattle sampled in our study. This disparity in supplier numbers as well as the lower than anticipated *Salmonella* LN prevalence might have hindered our ability to detect a statistically significant difference between the two groups. It is also crucial to consider the potential influence of other dietary components in the feeds, on-farm management practices, and external environmental factors, which could not be collected in the present study for the majority of suppliers, and could have affected the postbiotic's effectiveness, thus contributing to the observed results. Additional research is necessary to investigate this and other pre-harvest intervention approaches to reduce *Salmonella* burden in cattle LN. In light of the growing concern surrounding the use of antimicrobials in veterinary medicine and agriculture, options such as integrating postbiotics or DFM into cattle diets presents a promising avenue for

enhancing pre-harvest interventions in beef production, and advancing sustainability efforts. To the best of our knowledge, this study (study 1) for the first time evaluated, via an observational study, the effectiveness of using a postbiotic product as a feed additive in cull dairy cattle production systems.

Following a significant regional difference in *Salmonella* LN prevalence and seasonal *Salmonella* serotype diversity in the LN reported in Study 1, a second study was performed to provide estimates of baseline prevalence and serotype diversity across multiple regions in the U.S. In this study, three additional processing plants were enrolled, and LN samples were collected from cull dairy cattle shipped to five commercial processing plants across four U.S. regions (Upper Midwest, Midwest, Southwest, and Northeast). The results for regional prevalence and *Salmonella* serotype diversity were similar to Study 1 except that for the second study, an association between season and region with *Salmonella* LN prevalence was observed. Interestingly, the complete absence of seasonal patterns in *Salmonella* LN prevalence in the first study and the observed association between *Salmonella* prevalence and region only during colder seasons (Winter and Spring) in the second study was rather surprising since prior investigations have consistently reported increased *Salmonella* prevalence in warmer seasons. We hypothesize that the widespread belief of higher *Salmonella* prevalence in summer months may have fueled the efforts adopted by cattle farmers and the industry to tailor most pre-harvest intervention strategies and practices specifically to these warmer seasons. As such, there is a need to rethink *Salmonella* prevalence in other seasons particularly for the contributions of cull dairy cattle to the spread of *Salmonella* in ground beef. Additionally, the observed seasonal patterns in the probability of isolating a specific *Salmonella* serotype is a compelling area for further research. This warrants an in-depth exploration of the direct and indirect effects of season on animal

management practices, environmental conditions, and variations in cattle-related activities, all of which could contribute to the increased prevalence of certain distinct *Salmonella* serotypes in cull dairy cattle.

*Salmonella* LN prevalence in the Southwest region was higher in both studies (Chapter 2), which comes in agreement with previous observations. While increased environmental temperatures have historically been considered the primary driver for these prevalence estimates, findings from both studies (Chapter 2) indicate that the underlying causes may extend beyond climatic conditions as certain farms within the Southwest region were observed to contribute more to the *Salmonella* prevalence estimates than others. This observation confirms that there is a high variability in prevalence across farms irrespective of the region. We hypothesize that this outcome may be linked to differences in cattle environment, as well as differences in hygiene practices or other routine management procedures among cattle farms within that region. Furthermore, the overall high *Salmonella* LN prevalence observed could also be connected to the higher prevalence of non-mammalian vectors, particularly flies that are densely populated in the Southwest region. These vectors play a critical role in the transdermal transmission of *Salmonella*, eventually targeting the LN as a niche. Future studies should investigate the interaction between these vectors and seasons on *Salmonella* LN prevalence in the U.S., particularly in the Southwest region. Overall, the observed effects of seasonality on the association between *Salmonella* LN prevalence and regions, the inconsistent pattern in the seasonal probability of isolating a unique *Salmonella* serotype, and the substantial contributions of specific farms to the overall prevalence in the Southwest region collectively suggests that seasonal and regional variations may not be solely dependent on ambient temperatures, as other

environmental factors that change with season and are unique to certain regions may also contribute to these variations.

While seasonal and regional effects are still considered the leading risk factors affecting *Salmonella* LN prevalence and serotype diversity, data presented in both studies contributes to our understanding of how these factors might influence *Salmonella* prevalence independently or in synergy with other factors. These findings will consequently allow for better planning and implementation of risk assessment models considering the wide-ranging inconsistency in results reported in the literature. Furthermore, these findings will also help decision-makers in the beef industry assess the cost and benefits of specific pre-harvest interventions leading to appropriate allocation of resources, targeting specific seasons and regions in the U.S. Public health authorities can enhance surveillance efforts and preventive measures aimed at reducing the number of human illnesses caused by this pathogen, and safeguarding the overall health of the public.

From a public health, one health, and epidemiological standpoints, data regarding the antimicrobial resistance of *Salmonella* isolates contributes to a better understanding of the increased virulence of *Salmonella* which makes infections harder to treat and thus, increases the overall risk of disease spread, severe illnesses, and potential death. In both studies presented in Chapter 2, antimicrobial susceptibility testing was conducted on all the recovered *Salmonella* isolates with the aim of assessing resistance patterns among *Salmonella* organisms prevalent in cull dairy cattle. Whereas a higher percentage of isolates demonstrated pan-susceptibility, a considerable number of *Salmonella* organisms showed resistance to at least one antimicrobial with some of these isolates demonstrating multidrug resistance (MDR) patterns. It is also important to note that serotypes of public health importance (Newport and Typhimurium) were

identified among the MDR isolates. Despite the consistently reported low prevalence of *Salmonella* organisms in cull dairy cattle compared to feedlot cattle across studies (reviewed in Chapter 1), cull dairy cattle are known as the major reservoirs of antimicrobial-resistant (MDR) isolates. Recently, cull dairy cows were implicated in approximately 48% of all the MDR isolates recovered. Potential reasons for this were attributed to the age and antimicrobial therapy administered to the animals prior to culling from the herd, leading to the overall increase in the MDR isolates. Considering this, it is reasonable to argue that while cull dairy cattle may not contribute significantly to the overall *Salmonella* prevalence estimates, the presence of public health significant serotypes as well as MDR isolates within this cattle population may warrant further investigation. Generally, the results obtained here are quite worrisome and demand the introduction of alternative treatment options.

As previously discussed, studies presented in Chapter 2 were subject to limitations, one of which was our inability to quantify *Salmonella* isolates at lower concentrations using the qPCR method, resulting in only three positive LN samples quantified. While qPCR offers high sensitivity, specificity, low cost, and rapid results (making it a valuable tool for diagnosing foodborne infections, especially in cases where early detection is critical), its high detection-limit, limits our ability to quantify pathogens at lower concentrations. Future studies should consider adopting other lower-cost assays or exploring alternative quantification methods like the *Enterobacteriaceae* count plate (Petrifilm) method, the most probable number (MPN) detection method, or the hydrophobic grid membrane filtration (HGMF) method.

In summary, beyond their contributions to the scientific field, the findings presented in this work supply valuable real-world data that can inform and guide decision-making processes

for various stakeholders, including veterinarians, food safety experts, livestock producers, and government agencies.

## 3.2 Conclusion

Despite our results pointing to the lack of effectiveness of the postbiotic in significantly reducing *Salmonella* prevalence in subiliac LN of cull dairy cattle, we established that *Salmonella* LN prevalence in cull dairy cattle depends on the region. We further determined that the association between region and *Salmonella* LN prevalence in cull dairy cattle depended on season. Additionally, *Salmonella* serotypes Montevideo, Mbandaka, and Muenster were the most frequently isolated serotypes and their presence significantly varied by season. These findings contribute to filling the data gaps regarding *Salmonella* LN prevalence in cattle (in multiple regions in the U.S.) prior to entering the food supply chain. These estimates could be used to populate quantitative risk assessment models that are crucial to understanding the risk of human illness due to *Salmonella* that are attributed to cull cattle or ground beef products. Future research should prioritize a comprehensive exploration of the mechanisms that facilitate the entry and survival of *Salmonella* organisms within the lymphatic system. This enhanced understanding will be instrumental in identifying pre-harvest intervention strategies aimed at reducing the burden of *Salmonella* in cattle, which is pivotal for ensuring food safety and safeguarding public health.

# Appendix A - Study 1: Questionnaire deployed to dairy farmers supplying cattle to enrolled processing plants.

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Researchers at Kansas State University in collaboration with JBS and Diamond V are conducting a research study to evaluate whether a dietary intervention reduces the presence and concentration of *Salmonella* in LNs of cattle. The purpose of this questionnaire is to capture information regarding demographics, biosecurity, and management factors related to farms that culled cattle sent for harvest. **All the information gathered will remain confidential. For any questions or concerns, please contact Dr. Natalia Cernicchiaro (Kansas State University), Mrs. Sherri Trujillo (JBS) or Dr. Tom Edrington (Diamond V)**.

Contact information:
Natalia Cernicchiaro (KSU): ncernic@vet.k-state.edu, (office) 785.532.4241
Sherri Trujillo (JBS): sherri.trujillo@jbssa.com, (office) 970.506.8153
Tom Edrington (DV): tedrington@diamondv.com, (office) 979.571.4356

**Date:** Click or tap to enter a date.

**Farm Demographics**

The following questions are to obtain general demographics about your farm currently.

1. **Are your heifers raised off-site? ☐ Yes ☐ No**

- **If yes, in which region are they raised? (see Figure 1)** Select region from drop down list.

2. **How many milking cows does your farm have on-site currently?**

- ☐ <200 cows ☐ 200 to 2,000 cows ☐ >2,000 cows

3. **Does your farm use tie-stall housing? ☐ Yes ☐ No**

- **If no, ☐ dry lot, ☐ free-stall, or ☐ other:** Click here to enter text.

**Biosecurity/Management Factors**

The following questions are to obtain current biosecurity and other management factors that are employed on your farm.

4. **What material is used for cattle bedding?**

☐ **Sand, if so how frequently do you rake beds?**Click or tap here to enter frequency of raking (e.g., daily, every other day, weekly, etc.)

☐ **Dry lot, if so how frequently is manure removed?** Click or tap here to enter frequency of manure removal(e.g., daily, every other day, weekly, etc.)

☐ **Recycled compost, how often maintained?** Click or tap here to enter frequency of maintenance (e.g., daily, every other day, weekly, etc.)

☐ **Sawdust, how often maintained?** Click or tap here to enter frequency of maintenance (e.g., daily, every other day, weekly, etc.)

☐ **Other:** Click to enter other source of bedding used.**, how often maintained?** Click or tap here to enter frequency of maintenance (e.g., daily, every other day, weekly, etc.)

**How are pre-weaned calves (i.e., wet calves) housed on-site?**

☐ Individually ☐ Paired ☐ Grouped (3 or more)

6. **Do you perceive any risk of *Salmonella* introduction via any of the following pests on your farm? (Select all that apply)**

☐ Biting flies

☐ Rodents

☐ Birds

☐ Other, Click to enter other pests of concern.

7. **Do you currently have pest management strategies in place for the above pests? ☐ Yes ☐ No**

- **If yes, provide a brief overview of pest management strategies used:** Click or tap here to enter details on farm pest-management strategies employed. In example, mitigating biting flies with use of insecticide-impregnated ear tags.

8. **Do you utilize methods to decrease heat stress on-site? ☐ Yes ☐ No**

- **If yes, which methods are employed currently? (Select all that apply)**

☐ **Sprinklers**

☐ **Access to shade**

☐ **Fans**

☐ **Other:** Click to enter other heat stress management method.

9. **Do you currently use any products for the reduction/prevention of *Salmonellosis* (vaccines, DFMs, feed additives, antibiotics, etc.)?**

If yes, please list commercial names here.

**Cull Cattle Information**

The following questions refer to the current group of cattle that were sent to JBS for slaughter.

10. **What are the Top 3 production reasons for culling cattle today?**

- Click to enter production cull reason.
- Click to enter production cull reason.
- Click to enter production cull reason.

11. **What are the Top 3 disease reasons for culling cattle today?**

- Click to enter disease cull reason.
- Click to enter disease cull reason.
- Click to enter disease cull reason.

12. **How many cull animals (in this shipment) are represented by the following categories?**

| **Reason for culling** | **Heifer** | **Cow** | **Bull/Steer** |
|---|---|---|---|
| **Production** | Click here to enter the number of heifers culled today. | Click here to enter the number of cows culled today. | Click here to enter the number of bull/steers culled today. |
| **Disease** | Click here to enter the number of heifers culled today. | Click here to enter the number of cows culled today. | Click here to enter the number of bull/steers culled today. |

13. **For mature cows culled, if applicable, how many cows are represented by each <u>parity category:</u>**

| **Parity Category** | **Number of Mature Cows Culled** |
|---|---|
| **First Parity** | |
| **Second Parity** | |
| **Third Parity** | |
| **Fourth or Greater Parity** | |

14. **For mature cows culled, if applicable, which <u>stage of production</u> were they culled and what were the average days in milk (at the time of culling)?**

| **Stage of Production When Culled** | **Number of Mature Cows Culled** | **Average Days in Milk (DIM) When Culled** | **Range of DIM (Lowest-Highest)** |
|---|---|---|---|
| **Early Lactation** | | | |
| **Mid-Lactation** | | | |
| **Late Lactation** | | | |
| **Dry Period** | | | |

- 

**<u>Procurement Team, Data to Collect:</u>**

- **Date procured:** Click or tap to enter a date.
- **Farm ID:** Click or tap here to enter text.
- **Location:** Click or tap here to enter text.
- **BIFSCO region (see Figure 1):** Choose a region ID.
- **What predominant breed is reflected in this group of cull cattle?**

☐ Holstein ☐ Holstein-cross ☐ Other, Click here to enter breed.

- **Form completed by:**
  - **E-mail:** Click or tap here to enter text.
  - **Phone: (**XXX**)** XXX-XXXX
- **Procured cattle will be sent to which plant:**

**☐Tolleson, Arizona**

☐ **Souderton, Pennsylvania**

☐ **Green Bay, Wisconsin**

- **Number of cattle procured (# head sold to JBS):**

**Appendix A Figure A.1. Microbiological monitoring regions defined by BIFSCo.**