Prevalence of *Salmonella* in beef cattle lymph nodes and the role of iron uptake proteins on *Salmonella* growth and survival in food products

by

Aleicia Mushins

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract

Salmonella enterica subspecies Enteritidis and Typhimurium are the cause of most cases foodborne salmonellosis. *Salmonella* is predominately found in poultry, beef and dairy products. It is an intracellular pathogen, known to enter the lymphatic system and it has been found in the lymph nodes of beef and dairy cattle, as well as in ground beef. Lymph nodes at risk of entering the food chain are located throughout the body and include, but are not limited to the superficial cervical, subiliac, superficial inguinal, popliteal, and mandibular. When inside lymph nodes, *Salmonella* is protected from the adverse effects of antibacterial sprays and desiccation of dry chilling in meat processing facilities. Superficial cervical, subiliac, superficial inguinal and deep popliteal lymph nodes were tested to determine presence or absence of *Salmonella*. None of lymph nodes tested (0/39) contained detectable levels of *Salmonella*. Feed samples were tested to determine presence or absence of *Salmonella*, as feed is a known vector. None of the feed samples (0/16) contained detectable levels of *Salmonella*. More sampling needs to be done to determine if cattle processors in Alberta need to be concerned about lymphatic infection by *Salmonella*. This research tentatively concludes that <2.7% of lymph nodes in cattle from Alberta are positive for *Salmonella.*

Salmonella must survive in a broad range of environments: on vectors, in the gastrointestinal and lymphatic systems of cattle, on the food product, and in the GI tract of humans. A major factor in *Salmonella*'s ability to survive in a variety of different environments is its abundance of iron uptake systems. *Salmonella* has multiple ferrous iron uptake systems and it produces siderophores which uptake ferric iron. *S.* Enteritidis and *S.* Typhimurium and multiple iron uptake mutants of each serovar were used to determine the effect of each iron uptake gene on the growth and survival

of *Salmonella* in broth, ground beef and UHT milk. A bacteriostatic effect was observed when *S.* Typhimurium 3128 Δ*tonB*, *S.* Typhimurium 3128 Δ*iroNfepA*, or *S.* Enteritidis 3346 Δ*tonB* was grown in UHT milk (a low iron environment). Gene expression data also showed that *iroN* and *fepA* were among the genes most upregulated in iron deficient environments. It may be possible to use an antimicrobial that interacts or blocks TonB or both IroN and FepA to prevent the growth of *Salmonella* in low iron foods. This strategy may be very important for preventing *Salmonella* infection.

Preface

The research conducted for this thesis forms part of a research collaboration, led by Dr. Moussa Diarra, with Dr. Tineke Jones being the lead collaborator with the University of Alberta. The mutant strains were sent by Dr. Diarra and this thesis contributes to work done at Agriculture and Agri-Foods Canada. Figure 1 is based on an unpublished figure designed by Dr. M. Diarra. This is an original work by Aleicia Mushins. No part of this thesis has been previously published.

Acknowledgements

Thank you to my Mom and Dad for all your support. You both have helped me become the person I am today. Thank you, Luke, for always encouraging me to pursue my dreams even if they take me far away from you. Thank you, Tamara for being my best friend, travel partner and confidant.

Thank you to my amazing supervisors, Dr. Lynn McMullen and Dr. Tineke Jones, for believing in me. Thank you, Dr. McMullen, for giving me so many opportunities as a graduate student, especially as a teaching assistant. Thank you, Dr. Jones, for always keeping me on track, and reminding me to think about the questions I am trying to answer. Thank you, Dr. Ganzle for being on my committee and for being the chair of my defense.

Thank you to everyone in 2-50, without all of you I would not have had such a wonderful experience as a graduate student. Thank you, Oanh Nguyen, Chandré Van De Merwe, Danielle Schultze, and Yuan Fang for coffee breaks, adventures, climbing, and for making office work delightful and full of puns. Thank you to Danielle Balay for always taking time to answer my questions and for all the help you provided me with RNA isolation.

I want to thank MEaTnet for providing funding during my degree and internship. Thank you for providing many hands-on opportunities in the meat industry.

Finally, thank you to anyone in my life that I did not mention by name, there just isn't enough space here to properly thank everyone, but I could not have done this without you.

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List of Abbreviations

BGS	Brilliant green sulfa
BPW	Buffered peptone water
BS	Bismuth sulphite
CFU	Colony forming unit
CPI	Consumer price index
C_T	Cycle threshold
DC	Dendritic cell
DHBS	2,3-dihydroxybenzoylserine
Fur	Ferric uptake regulator
GI	Gastrointestinal
MAC	MacConkey No. 3
MkTTn	Muller-kauffmann tetrathionate-novobiocin
OD	Optical density
O/N	Overnight
RIN	RNA integrity values
RVS	Rappaport-Vassiliadis soya peptone
SCV	*Salmonella* containing vacuole
Sif	*Salmonella*-induced filaments
SPI1	*Salmonella* pathogenicity island 1
SPI2	*Salmonella* pathogenicity island 2
TS	Tryptic soy
T3SS	Type 3 secretion system
USDA	United States Department of Agriculture
WT	Wildtype
XLD	Xylose lysine deoxycholate

1. INTRODUCTION AND LITERATURE REVIEW

Decontamination procedures used in abattoirs such as lactic acid washes, spray chilling and dry chilling, lower the risk of *Escherichia coli* contamination in ground beef, but the same results are not observed for *Salmonella* (1). Decontamination procedures are successful in reducing the number of *Salmonella* on carcasses and whole muscles, but the United States Department of Agriculture (USDA) reported there has been no reduction of *Salmonella* in ground beef in recent years and the incidence of foodborne illness has remained relatively static (2). This finding is likely due to the lifestyle of *Salmonella*. It is an intracellular pathogen and can survive inside macrophages that reside through-out the lymphatic system. *Salmonella* living intracellularly are hidden from the adverse effects of antibacterial sprays such as lactic acid. Macrophages congregate inside lymph nodes where they can more easily activate the other cells of the immune system (3). Lymph nodes are located all over the body and vary in size and shape. Lymph nodes, especially deep tissue lymph nodes, are difficult and time consuming to excise from the surrounding adipose tissue during meat production (1). Adipose tissue is added to the muscle to attain the 10-30% fat expected in retail ground beef. Adipose tissue and the lymph nodes within it then become a vector for *Salmonella.*

Since *Salmonella* can survive intracellularly within cattle it can be transferred to food products, such as ground beef and milk. *Salmonella* also need to survive in these food products to eventually cause illness in humans. Bacteria need several nutrients for optimal growth and survival in the body and in food products. Iron is one of these essential nutrients as it is involved in DNA replication, oxidative stress protection and energy generation (4). Bacteria can sense the amount of available iron and in times of iron starvation, upregulation of iron sequestering and uptake proteins occurs (4). *Salmonella* have a number of iron uptake systems that act to increase the chances of intracellular

and extracellular survival (5). Without these systems, *Salmonella* would be incapable of obtaining the iron necessary for survival. Iron uptake proteins have been suggested as targets for antimicrobials for medicinal purposes in the cases of infection (6). However, using antimicrobials that target iron uptake proteins to limit bacterial growth and survival in food is not well studied. This research explored which iron uptake proteins are necessary for *Salmonella*'s survival in food products.

1.1 *Salmonella*

Salmonella is a facultative anaerobic, rod-shaped member of the family *Enterobacteriaceae* (7). *Salmonella* includes two species: *Salmonella enterica* and *Salmonella bongori*, and over 2400 serovars (8). These serovars have different host specificities and disease outcomes; for example, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Salmonella enterica* subsp. *enterica* serovar Enteritidis have unrestricted host specificity and cause different diseases depending on the species infected (7). Both serovars are asymptomatic in cattle and poultry, and cause enterocolitis in humans and swine. Asymptomatic infection in cattle and poultry allows these serovars of *Salmonella* to travel from farm to fork without detection. *Salmonella* is predominantly transferred via the fecal-oral route, usually by consuming contaminated water or food sources (7).

Salmonella was responsible for 7,731 reported illnesses and 17 deaths in Canada during 2015 (9). Since under reporting of food borne illnesses is a problem, researchers use modifiers to account for unreported illnesses and have estimated the number of cases to increase to 87,510 people/year when including unreported cases in Canada (10). This accounts for only a small portion of the estimated 93.8 million individuals worldwide that develop nontyphoidal

salmonellosis each year, and of these people, an estimated 155,000 cases result in death (11). Disease in humans is usually self-limiting enterocolitis that begins 12 to 72 h after ingestion and usually subsides within one week. However, complications can arise, and children, the elderly, and immunocompromised individuals are among those with the highest risk of severe illness and in some cases sepsis that can lead to death. Sequalae, such as reactive arthritis, have been observed in 6.4% and 15% of patients that have had infections caused by *S.* Typhimurium and *S.* Enteritidis, respectively (12).

1.2 Mode of infection and survival in cattle lymph nodes

Epidemiological studies indicate the main sources of *Salmonella* in cattle herds are contaminated feed and water, but contamination can also occur by introducing subclinically infected animals into a healthy herd (13). Some serovars of *Salmonella* such as *Salmonella* Montevideo, Anatum and Senftenberg can also be spread to herds by wildlife and biting flies due to unrestricted host specificity (14). Once ingested, *Salmonella* must survive through the rumen, reticulum, omasum, and abomasum before reaching the small intestine where infection commences. *Salmonella* localize to Peyer's patches, which are pockets of lymphoid-rich tissue in the ileum. *Salmonella* use fimbriae and other surface proteins to bind to host cells in the Peyer's patches. *Salmonella* is a facultative intracellular pathogen and after binding to host cells it penetrates the epithelial barrier using a variety of mechanisms. It can directly invade enterocytes, or it can be endocytosed by dendritic cells (DCs), which are immune cells that break cellular junctions and enter the intestinal space. It can also enter directly through M cells, which are immune cells that transfer antigens from the intestinal lumen to the underlying gut-associated lymphoid tissue and include macrophages (15). Additionally, *Salmonella* can pass through interstitial spaces between

enterocytes where there is cellular damage due to prolonged infection or other diarrheal disease (16). After passing through the epithelial cells, *Salmonella* preferentially infect macrophages.

Salmonella pathogenicity island 1 (SPI1) encodes the necessary type three secretion system 1 (T3SS-1) and effectors involved in invasion of host cells (17). The effectors are injected into the host cells and cause actin rearrangement that results in membrane ruffling and uptake of the *Salmonella* into *Salmonella* containing vacuoles (SCVs) (17). Mutations to the T3SS-1 structure and translocation genes of *S. enterica* serovar Dublin attenuates virulence in cattle (16). Another T3SS is needed for *Salmonella* to survive intracellularly. Once in a SCV, two-component regulatory systems sense the changed environment and regulates transcription of the genes on SPI2. T3SS-2 is located on SPI2 and releases effectors that polymerize to form *Salmonella*-induced filaments (Sifs) that protrude from the SCV and prevent lysozyme binding (18). Mutations in *sifA* result in impaired replication inside macrophages *in vivo* (19). The immune system of the host relies on lysosomal degradation of *Salmonella* to make antigens available for the major histocompatibility complex (20). The presentation of antigens on the major histocompatibility complex is important in activating the adaptive immune system and since *Salmonella* protects itself inside SCVs it can continue infection without detection.

Most oral *Salmonella* infections in cattle become localized to the gut and mesenteric lymph nodes, but occasionally macrophages and DCs can travel to more distal locations through the blood stream or within the lymphatic vessels (2). Afferent lymph vessels carrying macrophages and DCs infected with *Salmonella* can drain into peripheral lymph nodes. If *Salmonella* enters the host via a transdermal route, either from biting flies or epidermal injuries, it can be carried by

immune cells to the nearest group of lymph nodes. After transdermal infection of calves, *Salmonella* can be recovered from region specific lymph nodes up to 8 d post infection (21).

1.3 Prevalence of *Salmonella* on carcasses and in lymph nodes

Salmonella prevalence inside lymph nodes has been well researched in the United States; however, data from Canada is lacking. Most studies up to 2012 were concerned with *Salmonella* prevalence inside mesenteric lymph nodes, which are not incorporated into any food products because they are rendered with the intestines (1). Lymph nodes at risk of entering the food chain are located throughout the body and include, but are not limited to the superficial cervical (prescapular), subiliac (prefemoral), superficial inguinal (mammary or scrotal), popliteal, and mandibular (1, 22). These lymph nodes are in adipose tissue and are difficult and time consuming to excise. Small abattoirs with slow output rates have increased opportunity to remove these lymph nodes than large abattoirs with set line speeds and outputs of thousands of carcasses a day.

Of the 1,140 lymph nodes from the flank and chuck adipose tissues, which are the subiliac and superficial cervical lymph nodes, 2.3% and 1.6%, respectively, were positive for *Salmonella* (1). Cull cattle have an overall *Salmonella* prevalence in lymph nodes of 2.46%, which is higher than those from feed lot cattle, with an overall prevalence of 0.7% (1). Koohmaraie et al. (2012) tested carcasses throughout production and found that 96% of hide (n=100), 47% of preintervention carcasses (n=100), 0% of postintervention carcasses (n=50), 18% of lymph nodes (n=100), 7.14% of trim (n=14), and only 1% of ground beef (n=60) samples were positive for *Salmonella* (23). The authors concluded that interventions currently used are successful at

removing *Salmonella* from the carcass; however, lymph nodes located in the trim can carry *Salmonella* though the food chain. Pulse field gel electrophoresis was performed for each of the isolates and the *Salmonella* isolated from ground beef had restriction digest patterns that resembled the *Salmonella* isolated from the pre-intervention carcasses, but not the *Salmonella* found in the lymph nodes. This indicates that the likely source of contamination of ground beef was the hide, not lymph tissue. Many studies have just collected and tested lymph nodes at the time of meat processing which fails to give a broad picture of if lymph nodes are the main vector for *Salmonella* found in ground beef.

The prevalence of *Salmonella* varies depending on the feedlot. Haneklaus et al. (2012) tested lymph nodes from cattle at 8 different feedlots and found that 0% (n=84), 88% (n=85), 40% (n=25), 4% (n=50), 24% (n=25), 43% (n=28), and 40% (n=10) of samples were positive for *Salmonella* (24). It has been hypothesized that cattle temperament, stress level, veterinary intervention, and/or environmental differences among cattle at each of the feedlots may contribute to the discrepancies among feedlots (24).

Other studies have shown that *Salmonella* prevalence is not affected by breed (25), but is affected by temporal and spatial factors (26). Webb et al. (2017) divided their sampling locations into three regions across the United States (26). Region A included: Colorado, Illinois, Indiana, Iowa, Kansas, Kentucky, Maryland, Missouri, Nebraska, Ohio, Pennsylvania, Virginia, and West Virginia; region B included: Arizona, New Mexico, Oklahoma, and Texas; and region C included: California, Nevada, and Utah. Overall, in cooler months, prevalence of *Salmonella* decreased to 2.4% (n=2,704) from 8.2% in warmer months (n= 2,746). Region B had the highest

prevalence, with 6.5% (n=551) and 31.1% (n=570) for feedlot cattle in cooler and warmer months, respectively. Feedlots in Mexico also have a high prevalence of *Salmonella*; 76% of subiliac lymph nodes (n=68) tested positive for *Salmonella* (27). This leads to the conclusion that geography and climate are likely the largest contributing factors that determine whether *Salmonella* prevalence in lymph nodes will be low or high. Overall, the warmer the year-round climate, the more likely *Salmonella* will be found within cattle lymph nodes. However, there is limited data on the prevalence of *Salmonella* in the lymph nodes of cattle raised in more northern climates, such as Alberta.

1.4 *Salmonella* outbreaks in ground beef and milk

Ground beef and milk are known sources of *Salmonella.* Ground beef that tests positive for *Salmonella* is not recalled immediately, unlike when ground beef tests positive for one of the 7 enterohemorrhagic strains of *E. coli* (serovars: O157, O26, O45, O103, O111, O121, and O145) (28) and recalls that occur after outbreaks can be very costly to both the meat and dairy industries.

Cargill Meat Solutions recalled over 23,339 pounds of ground beef in 2012 after an outbreak of *S.* Enteritidis caused illness in 49 individuals and hospitalization of 12 (29). According to the US Bureau of Labor Statistics CPI (consumer price index), ground beef prices were $3.502/pound in the US in September 2013, which translates to a net loss of over $80,000 of ground beef for Cargill Meat Solutions. In 2011, another outbreak of *Salmonella* Typhimurium was linked to ground beef and caused illness in 20 individuals (30). The strain isolated from ground beef obtained from retail stores and from patient's homes was resistant to many common antibiotics,

which may have increased hospitalization rates for this outbreak (30). *Salmonella* prevalence in ground beef in Canada is not well reported. A study in 2002 reported testing 1,002 packages of ground beef purchased from retail stores in Alberta, and found that 1.3% (13/1,002) packages were positive (31). This is lower than estimates from the United States which range from 2.4 - 4.3% depending on the source (32). Even though the prevalence is relatively low, the negative impacts of *Salmonella* make it worth finding new interventions and antimicrobials that can be used to prevent *Salmonella* from entering ground beef.

Raw milk has also been the cause of many *Salmonella* outbreaks. For example, from 2007-2012, 81 distinct foodborne illness outbreaks linked to raw milk occurred in the US, with 3% caused by *Salmonella* (33). One of the few studies in Alberta that determined the prevalence of *Salmonella* in dairy cattle tested 2,248 cattle from 750 different pools (combining cattle from the same herd) found *Salmonella* in 5 of the 750 pools (34). Salmonellosis from milk is usually attributed to raw milk consumption and not generally a problem for pasteurized or UHT milk products. However, there has been an increase in consumers requesting raw milk products, and *Salmonella* is one of the most commonly reported pathogens in raw milk (35).

1.5 Iron uptake systems of *Salmonella*

A major factor in the ability of *Salmonella* to survive in a variety of different environments is the abundance of iron uptake systems. There is redundancy in the systems, for example, ferrous iron is transported across the inner membrane by FeoB, MntH and the SitABCD system (36).

Ferric iron is bound by low molecular weight compounds called siderophores that have a high affinity for Fe^{3+}. *Salmonella* produce two catecholate siderophores: enterobactin and salmochelin. Enterobactin is a cyclic trimer of 1,2-dihydroxybenzoylserine and salmochelin is a c-glycosylated derivative of enterobactin that is unique to *Salmonella* (37). Production of salmochelin gives *Salmonella* an advantage in the gut. In response to infection with *Salmonella*, hosts release large quantities of the iron sequestering protein, Lipocalin-2 to counteract iron acquisition (35). Lipocalin-2 binds to enterobactin to limit access to iron; however, it is unable to bind salmochelin, which allows *Salmonella* to continue to acquire iron and grow during infection (38).

Salmonella has multiple strategies to gain access to iron and different iron uptake systems are responsible for the acquisition of iron from different sources. IroN can uptake the catecholate siderophores, enterobactin and salmochelin. FepA can also uptake enterobactin, and IroN, FepA and CirA are all able to uptake the stable breakdown products of enterobactin and salmochelin, 2,3-dihydroxybenzoylserine (DHBS) (36) (Figure 1). DHBS can have linear trimeric, dimeric or monomeric forms (36). Iron uptake is so important to *Salmonella* survival that they have evolved uptake systems for siderophores produced by other bacteria and fungi. Myxochelins, protochelin, amonabactins, and corynebactin are examples of siderophores that are not produced by *Salmonella* which enter the cell via the FepA, IroN, and CirA uptake proteins (Table 1). Ferrichromes are a group of hydroxamate siderophores produced by fungi and can be taken up via the FoxA and Fhu proteins (36, 39) (Table 1). Translocation of siderophores requires active transport through TonB-dependent outer membrane proteins, powered by proton-motive force from the interactions of the cytoplasmic membrane proteins TonB, ExbB, and ExbD (40).

Primary metabolites such as α-keto acids, and α-hydroxy acids can also bind iron less strongly than siderophores and can be used as pseudo-siderophores (41).

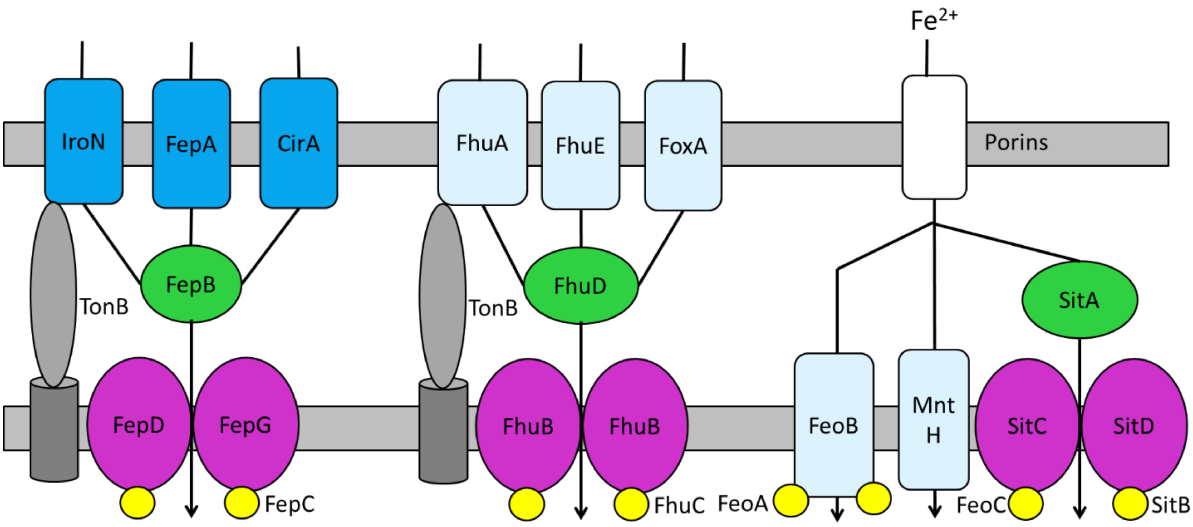

Figure 1. Overview of the iron uptake systems of *Salmonella.* TonB interacts with the outer membrane proteins: IroN, FepA, CirA, FhuA, FhuE and FoxA (not all are shown interacting in the diagram) (Based on an unpublished figure by Dr. M. Diarra).

Table 1. Catecholate, ferrichrome and ferrous iron uptake systems of *S. enterica* (36, 42, 43). Products (or the breakdown products) synthesized by *Salmonella* are bolded.

Proteins related to iron uptake	**Receptor for:**
FepA	**Enterobactin**
	DHBS
	Myxochelins
	Protochelin
IroN	**Enterobactin**
	Salmochelin
	DHBS
	Amonabactins
	Corynebactin
	Myxochelins
	Protochelin
CirA	**DHBS**
	Amonabactins
	Myxochelins
	Protochelin
FhuA and FoxA	Ferrichromes
FeoB	Ferrous iron

Many studies have indicated that iron genes are necessary for full virulence. Nugent et al. (2013) used *S.* Typhimurium WT and Δ*fepDGC* to determine if catecholate siderophores were necessary for survival in tomatoes (44). The deletion of *fepDGC* prevents IroN, FepA and CirA from translocating enterobactin and salmochelin across the cytoplasmic membrane. *S.* Typhimurium Δ*fepDGC* was unable to uptake iron efficiently and grew significantly less than the WT. However, after 7 d, the WT grew to 1.9 x 10^8 CFU ± 0.5 x 10^8 CFU and the Δ*fepDGC* mutant grew to 3.0 x 10^8 CFU ± 0.5 x 10^8 CFU. The numbers may be statistically significant but concluding that *fepDGC* is essential for growth within a tomato may not be a valid conclusion when there is such a minuscule difference between the growth of the WT and Δ*fepDGC* mutant. There is very little research on iron uptake genes and *Salmonella* survival in food products, but there is a large body of research that has reported the role of iron uptake genes and the ability of

Salmonella to colonize and survive through-out the murine, porcine, and avian intestinal tract, and in macrophages.

Rabsch et al. (2003) tested the ability of *S.* Enteritidis to colonize the cecum and liver of 4-day old chicks and found that Δ*fepA* and Δ*fepAiroN* mutants were able to colonize just as effectively as the WT (36). The authors concluded that intake of enterobactin and salmochelin were not necessary for full virulence of *S.* Enteritidis. *S.* Enteritidis Δ*fepAiroNcirA* was significantly attenuated in mouse serum (36). All the proteins encoded by these genes can uptake DHBS, concluding that it is most important for the bacteria to have a mode of ferric iron uptake, but it does not matter if it is able to uptake enterochelin breakdown products or the full siderophores.

Nagy et al. (2013) studied the effect of *fepB* in *S.* Typhimurium (45) and its ability to survive inside a murine macrophage, as macrophages tightly regulate iron. FepB is necessary to translocate iron from FepA, IroN and CirA across the cytoplasmic membrane. *S.* Typhimurium Δ*fepB* grew from 4.0 to 5.0 log CFU/mL over 22 h and the WT grew from 5.5 to 6.5 log CFU/mL over the same time. Initial cell counts were not done; however, it was mentioned that the WT and Δ*fepB* were added to the macrophage at a multiplicity of infection of 10. It is possible that both groups did not colonize the macrophages to the same extent, and both groups grew 1.0 log CFU/mL over 22 h. Although the authors concluded that *fepB* is required for *Salmonella* survival and replication within macrophages, the evidence provided does not support this conclusion. However, since macrophages tightly regulate intracellular and extracellular iron, siderophores are hypothesised to be of great importance for growth in these cells.

Other research has concluded that each of FeoB, MntH and the SitABCD system are essential for virulence of *S.* Typhimurium (46, 47). Although in all cases, none of the deletions prevented *Salmonella* survival, it did affect how quickly the bacteria were able to grow. Boyer et al. (2002) showed that in infections by S. Typhimurium Δ*sitABCD* and *S.* Typhimurium Δ*feoB*, 50% and 45% of mice (n=10), respectively, were able to survive intravenous infection longer than 30 d (47). Infections by *S.* Typhimurium WT, Δ*mntH*, and Δ*tonB* all resulted in 100% mortality of mice after 7 d infection. These findings suggest that uptake of ferrous iron by SitABCD and FeoB may be the most important factor for virulence in a mouse model.

Mutations in genes encoding various proteins in the iron uptake systems of *Salmonella* effect its survival to different extents. The redundancy of iron uptake systems means that *Salmonella* has many ways to uptake both ferrous and ferric iron, and not one single mutation has been shown to entirely prevent the survival of *Salmonella*. The environment that *Salmonella* is being tested in is an important factor. Some iron uptake proteins may be more important than others for survival in blood, different tissues or cells and in media or food matrices.

1.6 Ferric uptake regulator (Fur) gene regulation

Salmonella need to regulate intracellular iron levels very tightly as excess iron catalyses the formation of reactive oxygen species, which can cause cellular damage (48). Intracellular Fe^{2+} concentration transcriptionally controls iron uptake gene expression as it is a corepressor for a DNA-binding protein, Fur (ferric uptake regulator). Fur binds to a specific DNA sequence called the Fur box, a 19 bp inverted repeat sequence, and in most cases it acts as a repressor by inhibiting the binding of RNA polymerase (48). Fur regulates the expression of all the iron

uptake systems of *Salmonella* (6, 39, 49, 50). In low-iron conditions Fe^{2+} is not bound to Fur and repression ceases. Each operon regulated by Fur is repressed to a different extent, meaning that in low iron conditions some iron uptake genes will be expressed more than others.

1.7 Iron content in ground beef and milk

The iron content of ground beef and milk is listed in Table 2. Even though milk is a low iron food, it has enough iron for *Salmonella* to grow and survive. Iron is a micronutrient meaning bacteria require it in concentrations less than 10^{-4} M (5.58 µg/mL) (51). However, *Salmonella* have been known to grow in iron concentrations as low as 0.006 µg/mL (52).

Table 2. Iron content of ground beef and UHT milk. Based on information gathered by the USDA (53).

Food Product (%fat)	Iron content (µg/g)
Ground beef (7.2%)	23.2
UHT milk (2%)	0.286

1.8 Research objectives

The aim of this research was to inform the meat industry of potential risks of *Salmonella* contamination of ground beef by investigating the prevalence of *Salmonella* in cattle lymph nodes in Alberta, and to determine if infection is seasonally dependent. Another aim of this study was to determine which, if any, of the iron uptake proteins of *Salmonella* are essential for survival and/or growth in broth and food products with different iron concentrations. It is hypothesized that there is a very low prevalence of *Salmonella* in cattle lymph nodes in Alberta (<1%), similar to that of ground beef in Alberta, and that there will be seasonal variability with a higher infection rate in spring/summer. It was also hypothesized that deletions of iron uptake genes negatively affect

survival in a low iron food and broth, but not in a high iron food or broth. Additionally, it was hypothesized that in low iron conditions *Salmonella* will upregulate essential iron uptake genes.

The specific objectives were to determine:

1. the prevalence of *Salmonella* in cattle lymph nodes and feed,
2. the importance of different iron uptake proteins on the growth and survival of *Salmonella* in broth, ground beef and UHT milk,
3. and the expression of iron uptake genes in broth and UHT milk.

2. MATERIALS AND METHODS

2.1 Strains and growth conditions

The isolates, mutants and complements of *Salmonella enterica* subspecies *enterica* serovar Typhimurium ABBSB1218-1 #3128 and *Salmonella enterica* subspecies *enterica* serovar Enteritidis ABB07-SB3071 #3346 were provided by Dr. Moussa Diarra (Agriculture and Agri-Food Canada, Guelph, ON, Canada; Table 3). In this body of work these isolates will be referred to by the inventory numbers previously published (54). The mutants were generated by lambda red recombination and the complements were generated by using pSCA as a plasmid vector. All cultures were maintained at -80°C by adding equal parts culture in TS broth to a 50% glycerol-sterile water mix in 2 mL screw cap tubes (Starstedt, Nümbrecht Germany). Prior to each experiment strains were streaked onto Tryptic Soy [TS; Difco, Becton Dickinson (BD), New Jersey, USA] agar and incubated aerobically at 37°C overnight (O/N). Prior to use in experiments, individual colonies were picked from TS agar and inoculated into TS broth, which

was incubated at 37°C for 16 h. The strains with pSCA were grown in TS broth with 50 µg/mL kanamycin and streaked on TS agar with 50 µg/mL kanamycin to prevent the loss of the plasmid.

2.2 Lymph node collection, feed collection and sample processing

Lymph nodes (superficial cervical, superficial inguinal, subiliac, and deep popliteal) were collected from two provincial abattoirs in Alberta, Canada in 2016. Lymph nodes were collected from carcasses three days post-slaughter during meat fabrication. Only intact lymph nodes were collected; lymph nodes with minor incisions were discarded. Lymph nodes were collected in sterile sample bags (Whirl-Pak, Nasco, Fort Atkinson, WI, USA) and placed on ice for transportation.

Table 3. Strains of *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis wildtype (WT) and mutants used in this study.

Salmonella enterica subspecies *enterica* serovar:	WT or mutation
Typhimurium ABBSB1218-1 #3128	WT
	Δ*cirA*
	Δ*fepA*
	Δ*fhuA-CDB*
	Δ*iroN*
	Δ*feoAB*
	Δ*fhuA-CDB*Δ*cirA*
	Δ*fhuA-CDB*Δ*fepA*
	Δ*fhuA-CDB*Δ*iroN*
	Δ*fhuA-CDB*Δ*feoAB*
	Δ*tonB*
	Δ*tonB* + pSCA *tonB*
	Δ*iroN*Δ*fepA*
	Δ*fhuA-CDB*Δ*iroN*Δ*fepA*
	Δ*iroN*Δ*fepA* + pSCA *fepA*
	Δ*iroN*Δ*fepA* + pSCA *iroN*
	Δ*fhuA-CDB*Δ*iroN*Δ*fepA* + pSCA *fepA*
	Δ*fhuA-CDB*Δ*iroN*Δ*fepA* + pSCA *iroN*
Enteritidis ABB07-SB3071 #3346	WT
	Δ*cirA*
	Δ*fepA*
	Δ*fhuA-CDB*
	Δ*iroN*
	Δ*feoAB*
	Δ*fhuA-CDB*Δ*cirA*
	Δ*fhuA-CDB*Δ*fepA*
	Δ*fhuA-CDB*Δ*iroN*
	Δ*fhuA-CDB*Δ*feoAB*
	Δ*tonB*
	Δ*tonB* + pSCA *tonB*
	Δ*iroN*Δ*fepA*
	Δ*fhuA-CDB*Δ*iroN*Δ*fepA*

Samples were kept at 4°C and processed within 24 h of collection. Adipose was trimmed from each lymph node, and the lymph nodes were immersed in boiling water for 3 s using sterile forceps. Lymph nodes were weighed, placed into sterile petri dishes (Fisherbrand, Pittsburg, PA,

USA) on ice, and minced with a sterile scalpel. Feed samples (Table 4) were collected during 2016/2017 from bulk bins at a federal animal research center. All feed samples were collected in duplicate and randomly collected from different areas of the bins.

Table 4. Types of bovine feed sampled in 2016 and 2017 from an animal research facility.

Bovine Feed
16% Dairy cow lactation ration
Barley silage (3 years old)
Covered barley silage (3 years old)
Home rolled barley silage (2 years old)
90% barley silage with 10% ration and 10% grain
Ration 1 with silage
Ration 1

Minced samples and feed samples were transferred into a sterile stomacher bag (Seward BA6041, West Sussex, UK), and 20 mL of buffered peptone water (BPW; Oxoid, Hampshire, UK) were added for every 5 g of lymph node/feed sample. Samples were stomached (Seward Lab Blender 400/BA6021, West Sussex, UK) for 2 min. Each sample was incubated aerobically at 37°C O/N. After incubation, 0.5 mL and 0.1 mL of the sample was added to 10 mL of Muller-kauffmann Tetrathionate-novobiocin enrichment (MkTTn; Oxoid) broth, and Rappaport-Vassiliadis Soya Peptone enrichment (RVS; Oxoid) broth, respectively. Samples were incubated aerobically at 37°C O/N. All pre-enriched samples were streaked onto Bismuth Sulphite agar (BS; Oxoid), Brilliant Green Sulfa agar (BGS; Oxoid), and Xylose Lysine Deoxycholate agar (XLD; Difco), and incubated aerobically at 37°C O/N. Presumptive *Salmonella* colonies were streaked onto MacConkey No. 3 Agar (MAC; Difco) and incubated aerobically at 37°C O/N. Presumptive *Salmonella* colonies on MAC were streaked onto TS agar and incubated aerobically at 37°C O/N. Serological testing was performed using a *Salmonella* latex agglutination test kit

(FT0203, Oxoid). Positive colonies were confirmed by PCR using internal transcribed spacer primers [F-TAT AGC CCC ATC GTG TAG TCA GAA C, R- TGC GGC TGG ATC ACC TCC TT (55)]. PCR protocol was as follows: initial denaturation: 5 min, 94°C; denaturation: 30 s, 95°C; annealing: 30 s, 60°C; extension: 50 s, 72°C; 35 cycles; and final extension: 10 min, 72°C.

2.3 Growth of *S.* Typhimurium 3128 and *S.* Enteritidis 3346 WT and mutants

To determine differences in growth among the strains of *S.* Typhimurim and *S.* Enteritidis used in this study, growth curves were generated in TS broth. From the TS broth that had been incubated for 16 h, a 1% subculture was prepared by adding 50 µL of culture into 5 mL of fresh TS broth, then 200 µL of subculture was added in duplicate to wells of a 96 well plate. The starting cell concentration was approximately 7.0 log CFU/mL. OD_{600} was measured (Varioskan LUX multimode microplate reader, VL0000D0) every 2 h for 18 h at 37°C or plates were held at 8°C and measured after 0, 2, 4, 7, 18, and 41 d of storage.

To determine growth in the presence of an iron chelator, cells were washed three times by centrifugation at 5000 x g for 5 min at 23°C and re-suspended in TS broth containing 150 µM 2,2 dipyridyl (Sigma-Aldrich, ON, Canada). A 1% subculture was prepared in fresh TS broth with 150 µM 2,2 dipyridyl and incubated at 37°C for 16 h. A 1% subculture was prepared and 200 µL of subculture was added in duplicate to wells of a 96 well plate. OD_{600} was measured every 2 h for 18 h at 37°C.

2.4 Preparation of aseptic ground beef and % fat determination

Sirloin roasts were obtained from a federally inspected meat facility (each roast was used for one replication). Roasts were wiped with 100% ethanol and seared with a Bunsen burner. The external surface was cut away using a sterile scalpel blade. Roasts were then cut into cubes (2.5 cm^2) and placed inside a food processor (Kitchenaid, Kfp715) that had been wiped with ethanol. Beef was processed using the pulse setting until it reached desired consistency (meat showed evidence of protein extraction, but was not homogenized). Meat was kept on ice during the process. Ground beef was vacuum packaged and stored at -20℃ until use.

Frozen ground beef was freeze dried in 50 mL conical tubes (Starstedt, Nümbrecht Germany) for 7 d. Meat was weighed before and after freeze drying. Samples were ground (Waring Commercial Blender, 7011GC) and duplicate samples of 1.00 g of each sample was added to a thimble (Whatman high performance cellulose, 33 × 80 mm, 65 ml, Maidstone, UK). Fat was extracted using a Soxtec 2050 system (Foss, Hilleroed, Denmark) at 135℃ using the following parameters: boiling: 30 min, rinsing: 45 min, recovery: 10 min, pre-dry: 15 min. Cups containing samples were placed in an oven at 110℃ for 20 min, and in a desiccator for 20 min prior to being weighed. % fat was determined using the calculation: average weight of fat/ average weight of sample prior to desiccation x 100.

2.5 Growth and survival of *S.* Typhimurium 3128 and *S.* Enteritidis 3346 WT and mutants in ground beef

To determine the ability of *Salmonella* WT and mutants to grow in ground beef, a 1% subculture of each strain grown in TS broth was prepared and incubated for 18 h at 37℃, the cultures were

diluted 100-fold, and 0.1 mL of diluted culture was added to 10 g aseptically prepared ground beef (7.2% fat) to give a starting concentration of approximately 6 log CFU/g. Inoculated ground beef samples were vacuum packaged (FlairPak 5" x 7" vacuum pouches, Appleton, WI, USA; Multivac C 200 Tabletop chamber machine, Brampton, ON, Canada). Samples were processed after 0, 2, 6, 14, and 28 d of storage at 8°C. Vacuum sealed packages were opened using a sterile scalpel blade, 40 mL of BPW was added to the sample and the samples was stomached for 2 min. Dilutions were prepared and plated on TS agar incubated at 37°C O/N prior to enumeration.

To determine the ability of *Salmonella* and mutants to grow at 12.6°C in ground beef, an additional experiment was performed using a modified method. One gram of aseptically prepared ground beef (7.2% fat) was mixed with 1 mL sterile water to create a meat slurry. Samples were stored in 15 mL conical tubes (Starstedt, Nümbrecht Germany) at 12.6°C. Samples were processed after 0, 1, 2, 3, 4, and 10 d of storage. Dilutions were prepared and plated on TS agar and incubated at 37°C O/N prior to enumeration.

2.6 Survival of *S.* Typhimurium 3128 and *S.* Enteritidis 3346 WT and mutants in UHT milk

TS broth was individually inoculated with *Salmonella* strains (Table 2) and incubated for 18 hours at 37°C. A 1% subculture was prepared and incubated for 18 h at 37°C. Cells were washed twice by centrifugation at 5000 *x g* for 5 min at room temperature and re-suspended in 5 mL UHT milk (2% fat, Grand Pré, Terrebon, Quebec), the samples were diluted 100-fold, and 0.05 mL of diluted culture was added to 5 mL UHT Milk. Samples were processed after 0, 1, 2, 3, 4,

10, and 20 d of storage at 12.6°C. Samples were diluted, plated on TS agar and incubated at 37°C O/N prior to enumeration.

2.7 Expression of iron uptake genes by *S.* Typhimurium 3128 and *S.* Enteritidis 3346 WT in high and low iron environments using RT qPCR

To determine the which iron uptake genes are upregulated in low iron environments, *S.* Typhimurium WT and Δ*tonB* and *S.* Enteritidis WT and Δ*tonB* were grown in TS broth, TS broth supplemented with 150 μM 2,2 dipyridyl, and UHT milk. Each culture was incubated at 37°C until mid-log phase growth (approximately 3 h). RNAprotect cell reagent (Qiagen, MD, USA) was added to the culture (2:1), incubated at room temperature for 5 min, and centrifuged at 8,000 *x g* for 10 min. The supernatant was discarded, and cell pellets were frozen at -80°C for up to 1 week. RNA was isolated using RNeasy mini kit (Qiagen). RNA was eluted with 30 uL RNase-free water and quantified via spectrophotometry (Nanodrop One/One^c Microvolume UV-Vis, ND-ONE-W, Thermo Scientific, MA, USA) and quality was examined from the A260/A280 and A260/A230 ratios. RNA integrity numbers (RIN) were obtained by adding 1 μL of RNA to 5 μL RNA sample buffer (Agilent Technologies), sample was vortexed for 1 min, centrifuged for 10 s, heated at 72°C for 3 min, and kept on ice for at least 2 min. Sample was placed in the TapeStation 2200 (Agilent Technologies, CA, USA) and run on a RNA ScreenTape (Agilent Technologies).

DNase digestion was done using Dnase 1, Amp Grade (Invitrogen, CA, USA). cDNA synthesis was completed using Superscript III Reverse Transcriptase (Invitrogen) by adding 1 μg of RNA and random hexamer primers added to a final concentration of 5 ng/uL. qPCR was run using

Quantifast Sybr Green PCR kit (Qiagen). PCR protocol was as follows: initial denaturation: 5 min, 95°C; denaturation: 10s, 95°C; annealing and extension: 30s, 60°C; 40 cycles. Melt curves were done from 65-95°C. Each plate was run with controls containing no template and no reverse transcriptase. Standard curves were made by diluting cDNA of *S.* Typhimurium WT in TS broth 5-fold for a total of 5 dilutions. Primer efficiency was calculated using the equation $E = 10^{-1/slope}$. The slope was calculated using a semi log regression line plot of cycle threshold (C_T) values verses the log input of nucleic acid. A C_T value is the number of cycles needed for the fluorescent signal to exceed the background fluorescence. C_T values were determined by the program (StepOnePlus™ software). The relative gene expression data was analyzed using the Pffafl method with *rpoD* as a reference gene. Control conditions were WT grown in TS broth and the experimental conditions were either WT grown in TS broth with 2,2 dipyridyl or in UHT milk. Additionally, control conditions were Δ*tonB* grown in TS broth and experimental conditions were Δ*tonB* grown in TS broth with 2,2 dipyridyl.

Table 5. List of primers used in this study

Genes	Sequence (5'-->3')
cirA	ACGCATAGTTTTGCCGTTCCAG
	GTTTGCCTGGACACCAAACGAA
feoB	GTGGGGGCTGAATATCGCCTAT
	GGCGTAACAGGCTTAACACCAC
fepA	GCGTTTTGTCGAGGTTGCCATA
	TTTCAACGCGCCGGAACATAAA
fhuA (Typhimurium)	GAGGTGTAGAGCTGGAAGCCAA
	TGCTCTGGCGTATTCCCTTTCA
fhuA (Enteritidis)	TGTACGGGAAAAGTAGCCCTGG
	GGAACAGGCTGTCAGTACCCAT
iroN	TCCCGGACAATCCTTTGGTCTC
	CTCTCCGCTAACGTCAACTGGA
tonB	AATATTGACCACCAGCCCGGAA
	CGCAGCCAGCCAATATGTTTGA
rpoD	GGTCTGACCATCGAACAGGTGA
	CGCAAGTTCGCTTCAACCATCT

2.8 Statistics

Statistics were not performed for the experiment on prevalence in lymph nodes (described in section 2.2). All other experiments were repeated at least three times with duplicate samples taken within each replicate. All microbiological counts were converted to $\log_{10}$ (CFU/g or CFU/mL) prior to data analysis. Data was analyzed by the University Edition of Statistical Analysis Software using the generalized linear model procedure with repeated measurement analysis (Figure 10 and 13) or without repeated measurement analysis (Figures 2,3,5, and 6) and the analysis of variance procedure with one-way layout with means comparison (Figure 15-18). Tukey's test was used to determine differences among treatments.

3. RESULTS

3.1 *Salmonella* in lymph nodes and feed samples.

To determine the prevalence of *Salmonella* in bovine lymph nodes tissue, samples were taken during fall/winter (November-March) and spring/summer months (April-August). None of the 37 lymph nodes tested over the course of a year contained detectable amounts of *Salmonella* (Table 6).

Table 6. Number of *Salmonella* positive lymph node samples collected at different times through-out the year.

Lymph node	Collected November- March	Positive	Collected April-August	Positive
Superficial cervical	4	0	3	0
Superficial inguinal	16	0	5	0
Subiliac	8	0	0	0
Deep popliteal	0	0	1	0

Feed samples were also tested to determine if feed was a likely source of *Salmonella* contamination. None of the 16 bovine feed samples collected through-out the year contained detectable levels of *Salmonella* (Table 7).

Table 7. Number of *Salmonella* positive bovine feed samples collected at different times through-out the year.

Bovine feed samples	Collected November-March	Positive	Collected April-August	Positive
	9	0	7	0

3.2 Growth of *S.* Typhimurium 3128 WT and mutants in TS broth and TS broth containing 2,2 dipyridyl

To gain an understanding of the growth of *S.* Typhimurium 3128 WT and mutants, growth curves were generated by measuring the OD_{600} every 2 h during growth in either TS broth or TS broth containing 150 µM 2,2 dipyridyl. OD_{600} measurements after 4 h incubation at 37°C were compared (Figure 2). No differences were observed in the OD_{600} of the WT and mutants of *S.* Typhimurium 3128 grown in TS broth. Addition of the chelating agent 2,2 dipyridyl significantly decreased ($P<0.05$) the OD_{600} of *S.* Typhimurium 3128 WT and mutants when compared to growth in TS broth. *S.* Typhimurium 3128 Δ*tonB* had significantly lower OD_{600} ($P<0.05$) in TS broth containing 2,2 dipyridyl when compared to the WT and other mutants. None of the other mutations significantly influenced the growth of *S.* Typhimurium 3128 at 4 h.

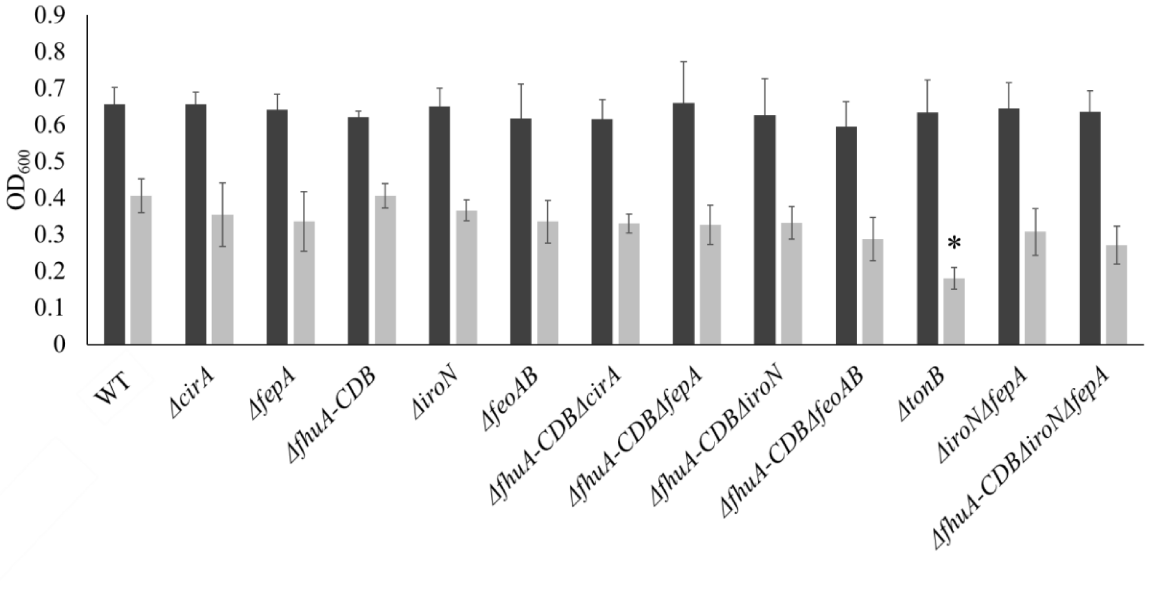

Figure 2. OD_{600} measurements of *S.* Typhimurium 3128 WT and mutants after 4 h incubation at 37°C in TS broth (■) and TS broth with 150 uM 2,2 dipyridyl (■). Initial OD_{600} measurements were below detection level. Data are means ± standard deviation of three replicates. **S**tatistically significant ($P<0.05$) differences among strains grown in the same media are indicated by an asterisk (*).

Growth curves of *S.* Typhimurium 3128 WT and mutants indicated that the Δ*tonB* mutant had the most gradual increase in numbers, as well as the second lowest OD_{600} during the stationary phase when compared to the other mutants and WT (Figure 3). *S.* Typhimurium 3128 Δ*fhuA-CDB*Δ*feoAB* had the lowest OD_{600} during the after 8 h incubation. Only the curves for the WT and select mutants are shown.

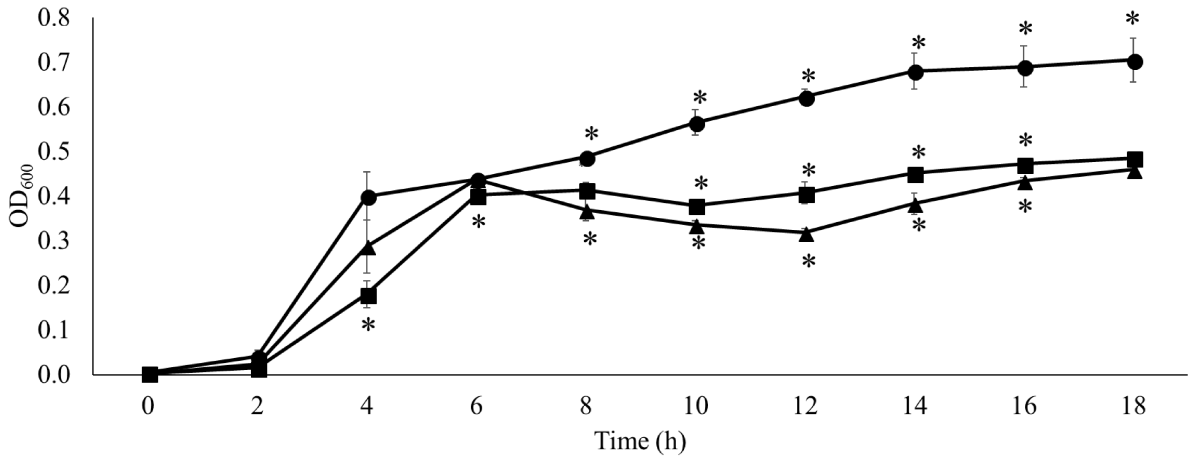

Figure 3. OD_{600} measurements of *S.* Typhimurium 3128 WT (●), Δ*fhuA-CDB*Δ*feoAB* (▲), and Δ*tonB* (■) grown in TS broth with 2,2 dipyridyl at 37°C. Data are means ± standard deviation of three replicates. **S**tatistically significant ($P<0.05$) differences between each mutant and the WT at each time are indicated by an asterisk (*).

To determine long term growth of *Salmonella* at refrigeration temperature (8°C), growth was determined over 41 days (Figure 4). No differences were observed in growth between *S.* Typhimurium 3128 WT and each of the mutants. *S.* Typhimurium 3128 reached the stationary phase after 18 d with an average OD_{600} of 0.52 ± 0.02.

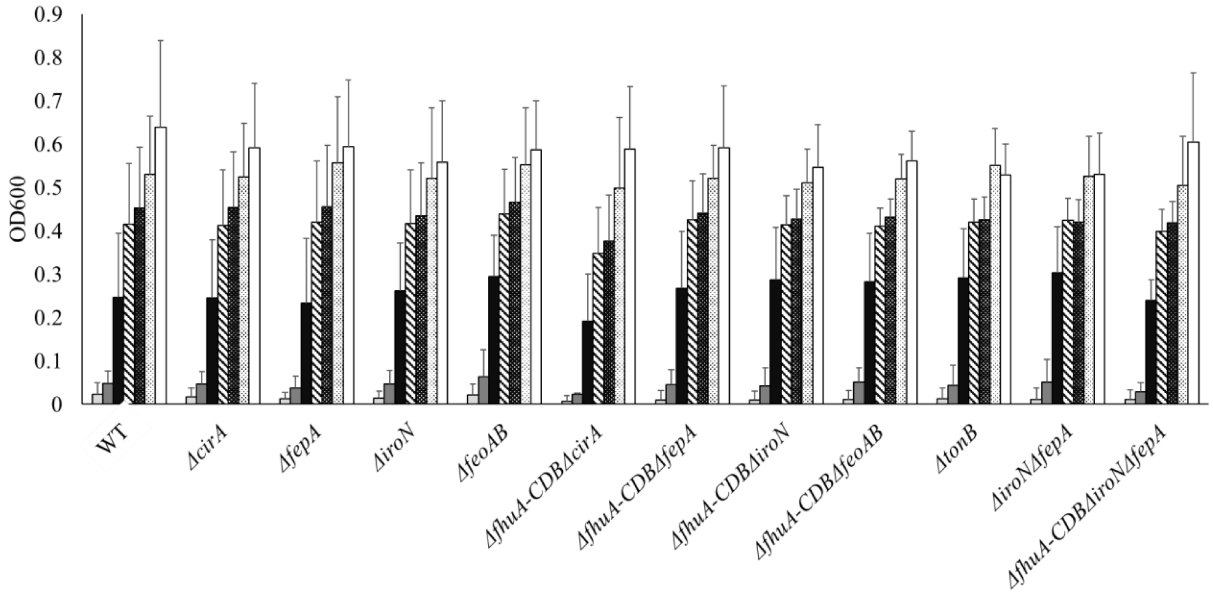

Figure 4. OD_{600} of *S.* Typhimurium 3128 WT and mutants in TS broth stored at 8°C for 0 (□), 2 (■), 4 (■), 7 (▨), 11 (▩), 18 (⁙), and 41 (□) d. Data are means ± standard deviation of three replicates.

3.3 Growth of *S.* Enteritidis 3346 WT and mutants in TS broth and TS broth containing 2,2 dipyridyl

To gain an understanding of the growth of *S.* Enteritidis 3346 WT and mutants, growth curves were completed by measuring the OD_{600} every 2 h of TS broth and TS broth containing150 µM 2,2 dipyridyl. OD_{600} measurements after 4 h incubation at 37°C were compared (Figure 5). No differences were observed in the OD_{600} between the WT and each of the mutants of *S.* Enteritidis 3346 grown in TS broth. Addition of the chelating agent 2,2 dipyridyl significantly lowered the OD_{600} of *S.* Enteritidis 3346 WT and mutants when compared to growth in TS broth. *S.* Enteritidis 3346 Δ*tonB* had significantly lower OD_{600} when compared to the WT and all other mutants grown in TS broth containing 2,2 dipyridyl. None of the other mutations significantly influenced the growth of *S.* Enteritidis 3346 at 4 h.

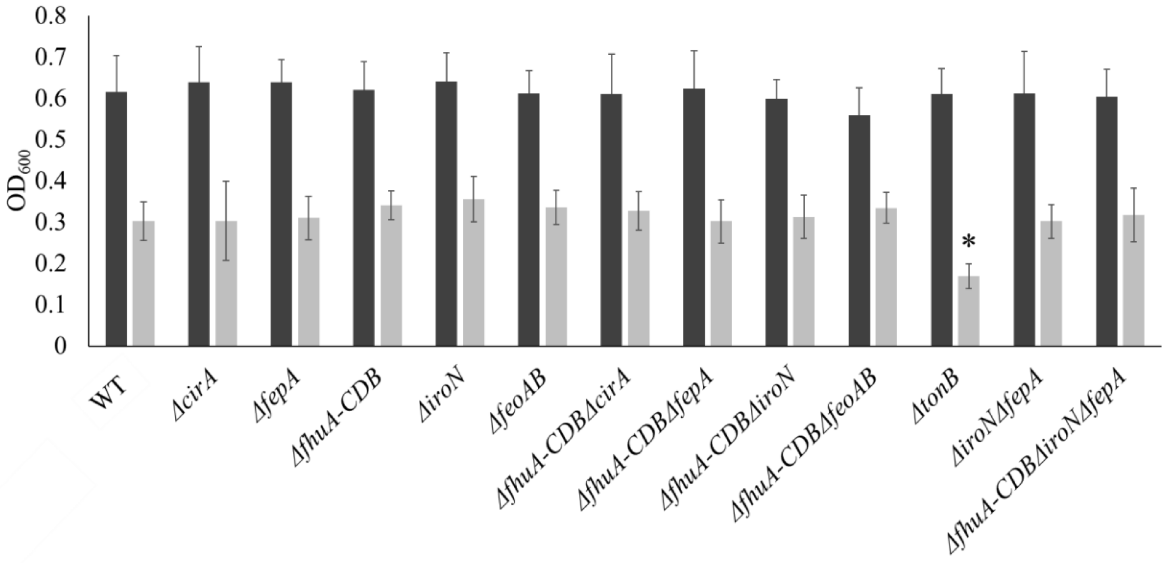

Figure 5. OD_{600} measurements of *S.* Enteritidis 3346 WT and mutants after 4 h incubation at 37°C in TSA (■) and TSA with 150 uM 2,2 dipyridyl (■). Initial OD_{600} measurements were below the detection level. Data are means ± standard deviation of three replicates. **S**tatistically significant ($P<0.05$) differences among strains grown in the same media are indicated by an asterisk (*).

Growth curves of *S.* Enteritidis 3346 WT and mutants indicated that Δ*tonB* had the most gradual increase in numbers, as well as the lowest OD_{600} during the stationary phase when compared to the other mutants and the WT (Figure 6). *S.* Typhimurium 3128 Δ*fhuA-CDB*Δ*feoAB* had the highest OD_{600} during the stationary phase after 12 h incubation. Only the data for WT and select mutants are shown.

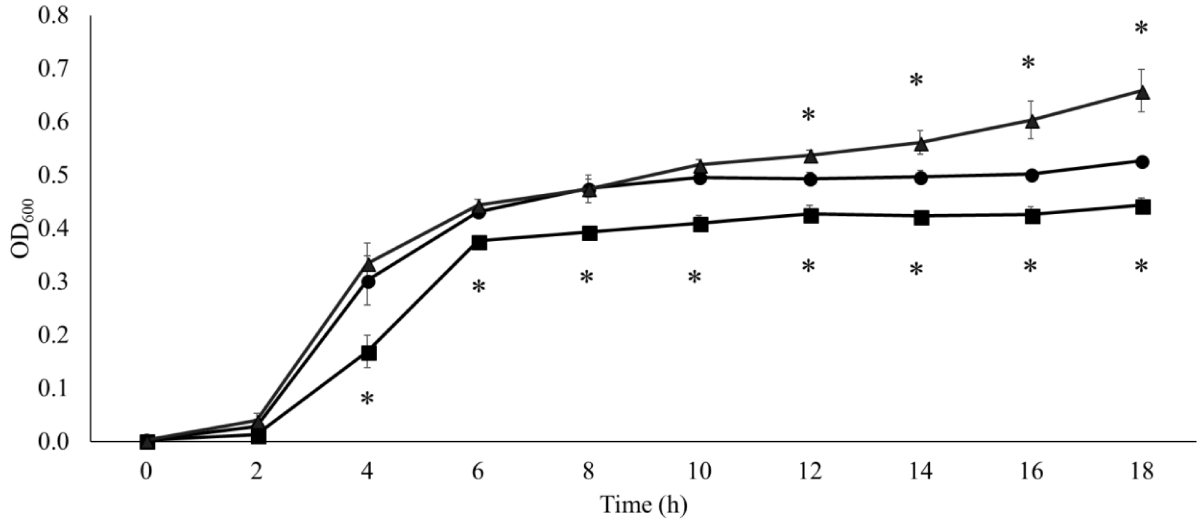

Figure 6. OD_{600} measurements of *S.* Enteritidis 3346 WT (●), Δ*fhuA-CDB*Δ*feoAB* (▲), and Δ*tonB* (■) in TS broth with 2,2 dipyridyl at 37°C. Data are means ± standard deviation of three replicates. **S**tatistically significant ($P<0.05$) differences between each mutant and the WT at each time are indicated by an asterisk (*).

To determine long term growth of *Salmonella* at refrigeration temperature (8°C), growth was determined over 41 d (Figure 7). No differences were observed in the OD_{600} between *S.* Enteritidis 3346 WT and each of the mutants. *S.* Enteritidis 3346 reached the stationary phase after 18 d, with an average OD_{600} of 0.62 ± 0.02.

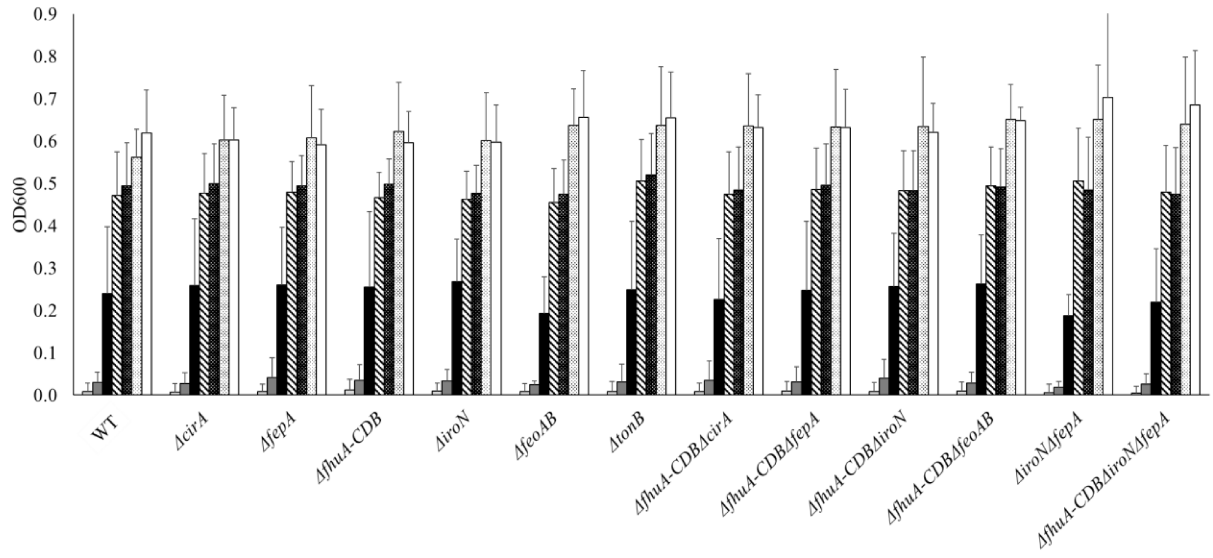

Figure 7. OD_{600} of *S.* Enteritidis 3346 WT and mutants in TS broth stored at 8°C for 0 (□), 2 (■), 4 (■), 7 (▨), 11 (▩), 18 (⁙), and 41 (□) d. Data are means ± standard deviation of three replicates.

3.4 Growth of *S.* Typhimurium 3128 WT and mutants in ground beef and UHT milk

To determine the importance of iron uptake proteins to the growth of *S.* Typhimurium 3128 in food products, ground beef (high iron) and UHT milk (low iron) was inoculated with *S.* Typhimurium 3128 and each of the mutants. Inoculated ground beef was stored at 8°C for 28 d or at 12.6°C for 10 d. UHT milk was stored at 12.6°C for 20 d. No differences were observed between the cell counts of the WT or any of the mutants of *S.* Typhimurium 3128 during the 28 d of storage (Figure 8).

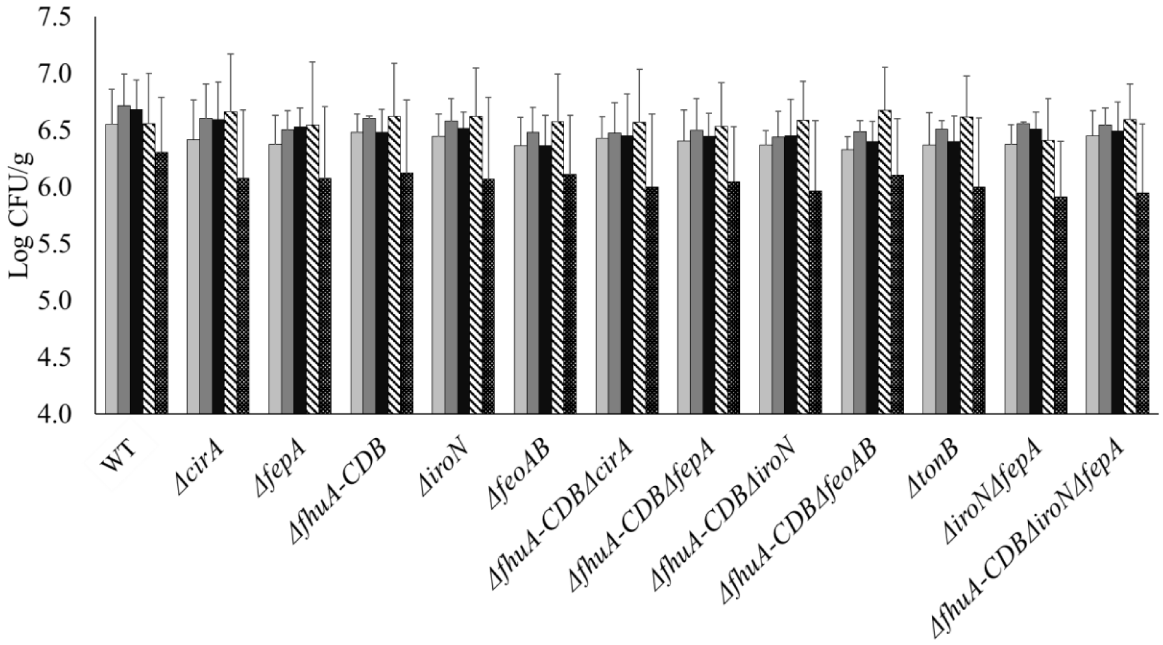

Figure 8. Counts of *S.* Typhimurium 3128 WT and mutants in ground beef stored at 8°C for 0 ( ), 2 ( ), 6 ( ), 14 ( ), and 28 ( ) d. Data are means ± standard deviation of three replicates.

Storage at 12.6°C allowed for growth of *S.* Typhimurium 3128 in ground beef during 10 d of incubation; however, no differences between the cell counts of the WT or any of the mutants (Figure 9).

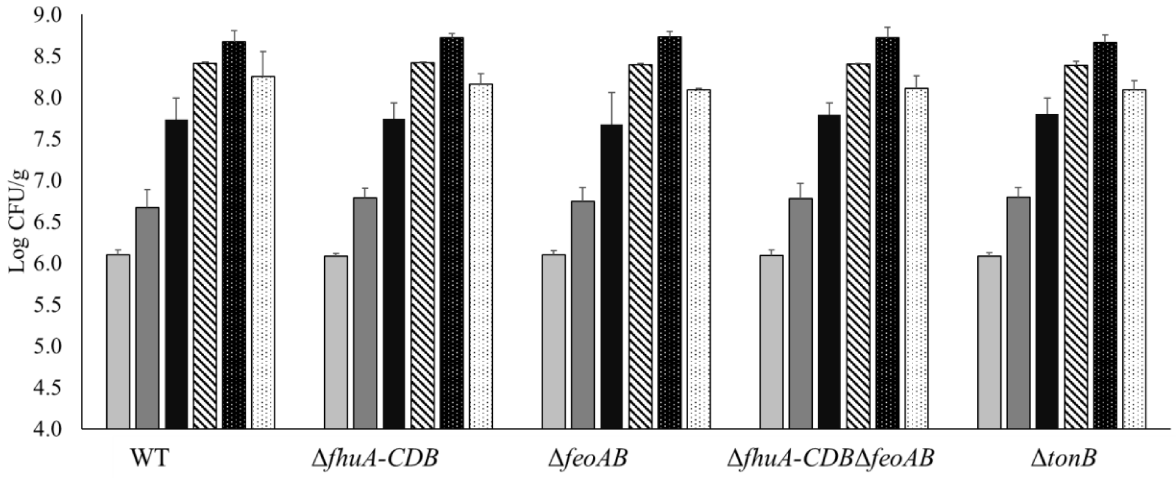

Figure 9. Counts of *S.* Typhimurium 3128 WT and mutants in ground beef stored at 12.6°C for 0 ( ), 1 ( ), 2 ( ), 3 ( ), 4 ( ), and 10 ( ) d. Data are means ± standard deviation of three replicates.

UHT milk was inoculated with *S.* Typhimurium 3128 WT, mutants, and complements. There were significant differences in the growth of *S.* Typhimurium 3128 Δ*fhuA-CDB*Δ*iroN*Δ*fepA*, *S.* Typhimurium 3128 Δ*iroN*Δ*fepA, S.* Typhimurium 3128 Δ*tonB* (Figure 10) after 3 d of storage. The WT and all other mutants continued to grow, while there was a bacteriostatic effect observed for the Δ*fhuA-CDB*Δ*iroN*Δ*fepA,* Δ*iroN*Δ*fepA* and Δ*tonB* mutants. All the complements with pSCA insertions, with the exception of PSCA-*tonB,* started with lower initial inoculum levels, but ended up growing to numbers similar to that of the WT.

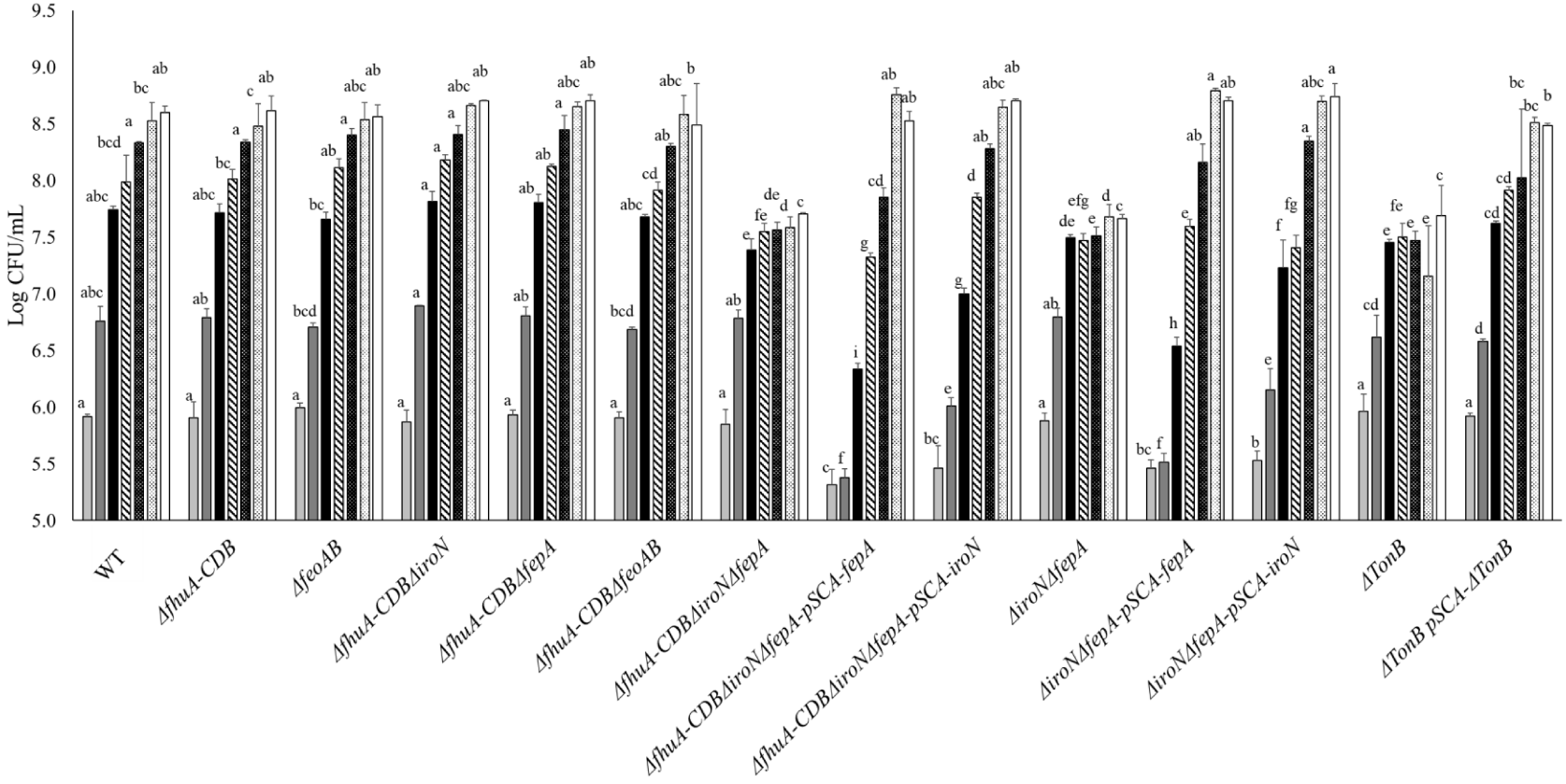

Figure 10. Counts of *S.* Typhimurium 3128 WT and mutants in UHT milk stored at 12.6°C for 0 (□), 1 (▒), 2 (■), 3 (▨), 4 (▩), 10 (▩), and 20 (□) d of storage. Data are means ± standard deviation of three replicates. Bars for each storage time with different letters indicate significant differences among strains ($P<0.05$).

3.5 Growth of *S.* Enteritidis 3346 WT and mutants in ground beef and UHT milk

Ground beef and UHT milk were inoculated with *S.* Enteritidis 3346. Ground beef was stored at 8°C for 28 d or at 12.6°C for 10 d. UHT milk was stored at 12.6°C for 20 d. No differences were observed between the cell counts of the WT or any of the mutants of *S.* Enteritidis during the storage of ground beef for 28 d at 8°C (Figure 11). Due to the slow growth at 8°C, subsequent experiments with ground beef and UHT milk were carried out at 12.6°C. Only select mutants were included in subsequent experiments based on differences in growth in TS broth at 8°C.

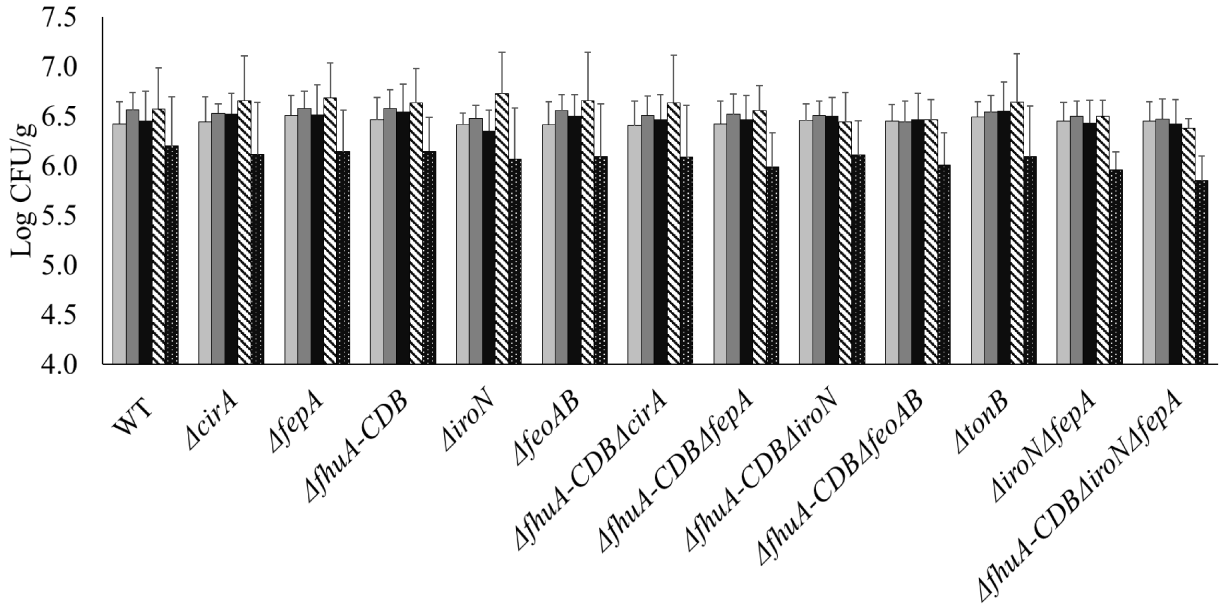

Figure 11. Counts of *S.* Enteritidis 3346 WT and mutants in ground beef stored at 8°C for 0 (□), 2 (▒), 6 (■), 14 (▨), and 28 (▩) d. Data are means ± standard deviation of three replicates.

Increasing the temperature to 12.6°C allowed growth of *Salmonella* in ground beef over 10 d of storage; however, no differences were observed between the cell counts of the WT or any of the mutants of *S.* Enteritidis during the 10 d of storage (Figure 12).

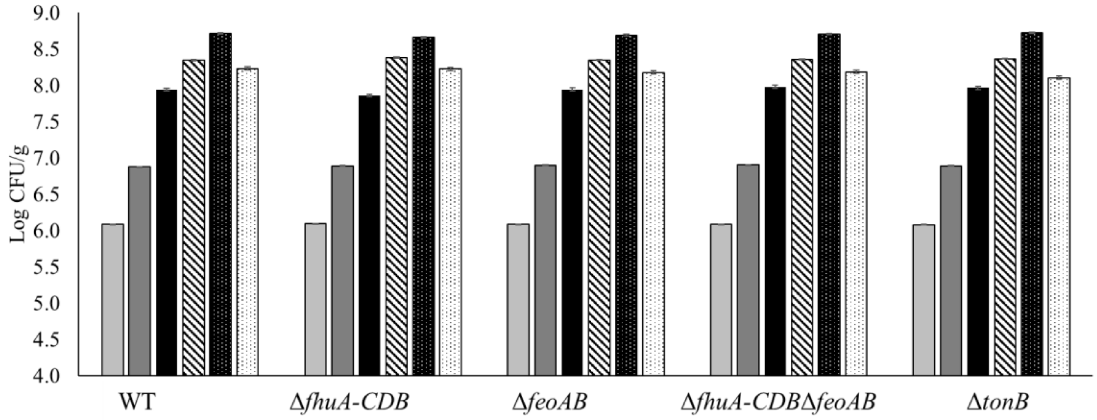

Figure 12. Counts of *S.* Enteritidis 3346 WT and mutants in ground beef stored at 12.6°C for 0 ( ), 1 ( ), 2 ( ), 3 ( ), 4 ( ), and 10 ( ) d. Data are means ± standard deviation of three replicates.

UHT milk was inoculated with *S.* Enteritidis 3346 WT, mutants, and complements. There were significant differences among the growth of *S.* Typhimurium 3128 Δ*tonB* and the WT and other mutants (Figure 13). After three days of storage, the counts of *S.* Typhimurium 3128 Δ*tonB* remained relatively static, while the WT and other mutants continued dividing and cell counts increased to a maximum of 8.5 to 9.0 log CFU/mL.

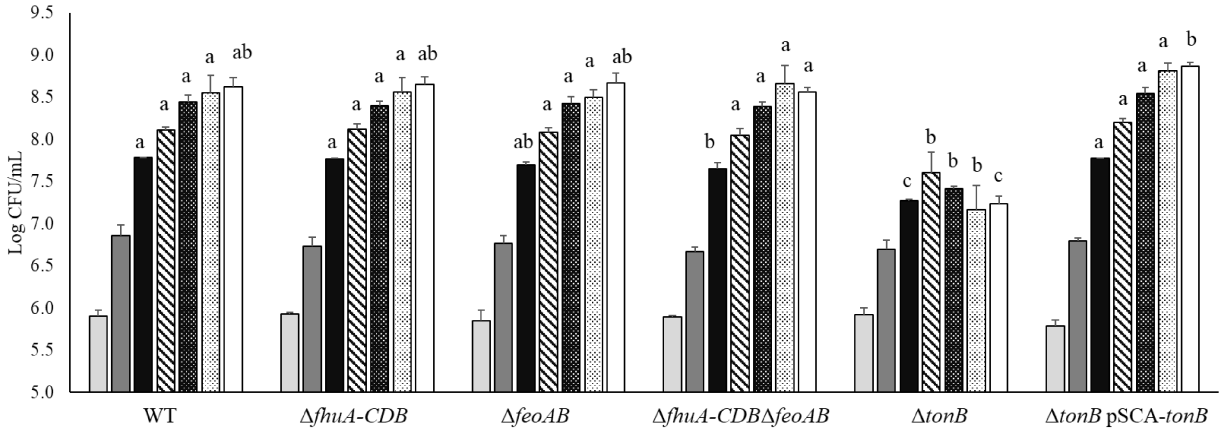

Figure 13. Counts of *S.* Enteritidis 3346 WT and mutants in UHT milk at 12.6°C for 0 (□), 1 (▒), 2 (■), 3 (▨), 4 (▩), 10 (⬚), and 20 (□) d of storage. Data are means ± standard deviation of three replicates. Letters indicate significant differences among counts for each mutant on the same day of storage ($P<0.05$).

3.6 Expression of iron uptakes genes by *S.* Typhimurium 3128 and *S.* Enteritidis 3346

To determine the extent of upregulation of selected iron uptake genes, relative gene expression was determined. Quality of RNA was determined by A260/A280, A260/A230, and RIN values (Table 8). RNA isolated from TS broth and TS broth supplemented with 2,2 dipyridyl had concentrations that exceeded or far exceeded 100 ng/µL and had A260/280 and A260/230 ratios around 2.0. RIN values were above 8 in all cases indicating good quality RNA. RNA isolated from UHT milk had very low concentrations of less than 12 ng/µL and low A260/280 values around 1.7. At such low concentrations A260/230 values and RIN values are very inaccurate and therefore were not determined. Standard curves were made for each primer pair by plotting C_T values verses the log input of nucleic acid. Standard curves are dose-response curves and determine if non-target cDNA is amplified. Different concentrations of cDNA result in proportionally inverse C_T values, and a slope of -3.32 is equal to a primer efficiency of 2. A standard curve (Figure 14) of the *feoB* primer pair was included which had an efficiency of 2.02. The efficiencies of all primer pairs are summarized (Table 9) and had acceptable efficiencies

ranging from 1.88 to 2.08. The primer pairs for the genes *fepA* and *tonB* had the lowest and highest efficiencies, respectively.

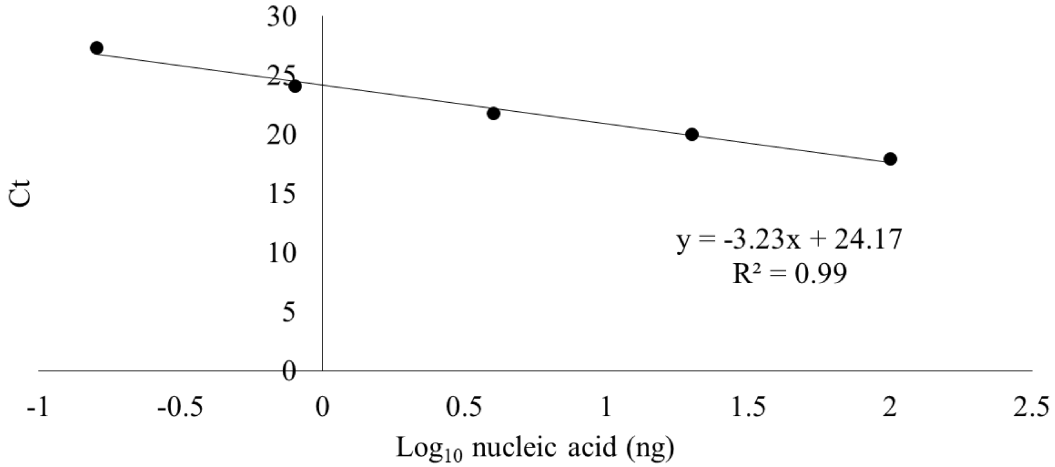

Figure 14. Standard curve made using C_T values verses log nucleic acid concentration for *feoB* primers.

Table 8. Concentrations and A260/280, A260/230, and RIN values of RNA isolated from different strains of *Salmonella* in different medias.

Replicate	Strain	Media	Concentration (ng/uL)	A260/280	A260/230	RNA integrity number (RIN)
1	*S.*Typhimurium	TS broth	814.0	2.16	2.20	8.3
	*S.*Typhimurium Δ*tonB*		658.9	2.09	1.99	9.3
	*S.*Enteritidis		692.4	2.09	2.27	9.2
	*S.*Enteritidis Δ*tonB*		676.0	2.10	2.13	9.6
	*S.*Typhimurium	TS + 2,2 dipyridyl	318.2	2.10	2.26	8.7
	*S.*Typhimurium Δ*tonB*		330.2	2.14	2.32	8.8
	*S.*Enteritidis		367.6	2.09	1.99	9.0
	*S.*Enteritidis Δ*tonB*		306.0	2.10	2.33	9.1
	*S.*Typhimurium	UHT milk	8.0	1.71	n/a	n/a
	*S.*Enteritidis		5.8	1.71	n/a	n/a
2	*S.*Typhimurium	TS broth	718.6	2.13	2.32	8.9
	*S.*Typhimurium Δ*tonB*		850.5	2.14	2.36	8.3
	*S.*Enteritidis		935.8	2.15	2.35	9.1
	*S.*Enteritidis Δ*tonB*		855.2	2.16	2.43	9.5
	*S.*Typhimurium	TS + 2,2 dipyridyl	385.0	2.11	2.10	8.7
	*S.*Typhimurium Δ*tonB*		184.1	2.10	1.34	8.7
	*S.*Enteritidis		294.5	2.10	2.04	8.9
	*S.*Enteritidis Δ*tonB*		179.4	2.10	1.87	9.3
	*S.*Typhimurium	UHT milk	11.4	1.73	n/a	n/a
	*S.*Enteritidis		7.4	1.57	n/a	n/a
3	*S.*Typhimurium	TS broth	962.9	2.13	2.48	9.0
	*S.*Typhimurium Δ*tonB*		985.6	2.16	1.99	8.3
	*S.*Enteritidis		1068.0	2.14	2.32	8.5
	*S.*Enteritidis Δ*tonB*		1035.2	2.15	2.49	9.1
	*S.*Typhimurium	TS + 2,2 dipyridyl	491.7	2.10	2.20	8.3
	*S.*Typhimurium Δ*tonB*		333.3	2.15	1.85	8.5
	*S.*Enteritidis		446.0	2.13	1.88	8.7
	*S.*Enteritidis Δ*tonB*		273.6	2.03	1.44	9.1
	*S.*Typhimurium	UHT milk	2.5	1.98	n/a	n/a
	*S.*Enteritidis		3.0	1.77	n/a	n/a

Table 9. qPCR primer efficiencies for primers used in this study. Primer efficiencies were calculated using the equation $E = 10^{-1/slope}$. The slope was calculated by plotting cycle threshold (C_T) values verses the log input of nucleic acid. R^2 values were determined by comparing the data points to the regression line.

Primers	Primer Efficiency (E)	R^2
cirA	1.96	0.99
feoB	2.02	0.99
fepA	1.88	0.99
fhuA (Typhimurium)	1.99	0.99
fhuA (Enteritidis)	2.02	0.99
iroN	2.05	0.99
tonB	2.08	0.98
rpoD	2.02	0.99

In TS broth with 2,2 dipyridyl, *S.* Typhimurium 3128 upregulated *fepA*, *iroN*, and *cirA* significantly more than *feoB* and *tonB* (Figure 15). In UHT milk, *S.* Typhimurium 3128 upregulated *fepA*, *cirA*, *iroN*, and *fhuA* more than *tonB* (Figure 16). *feoB* was not upregulated to the same extent as *fepA* or *iroN*.

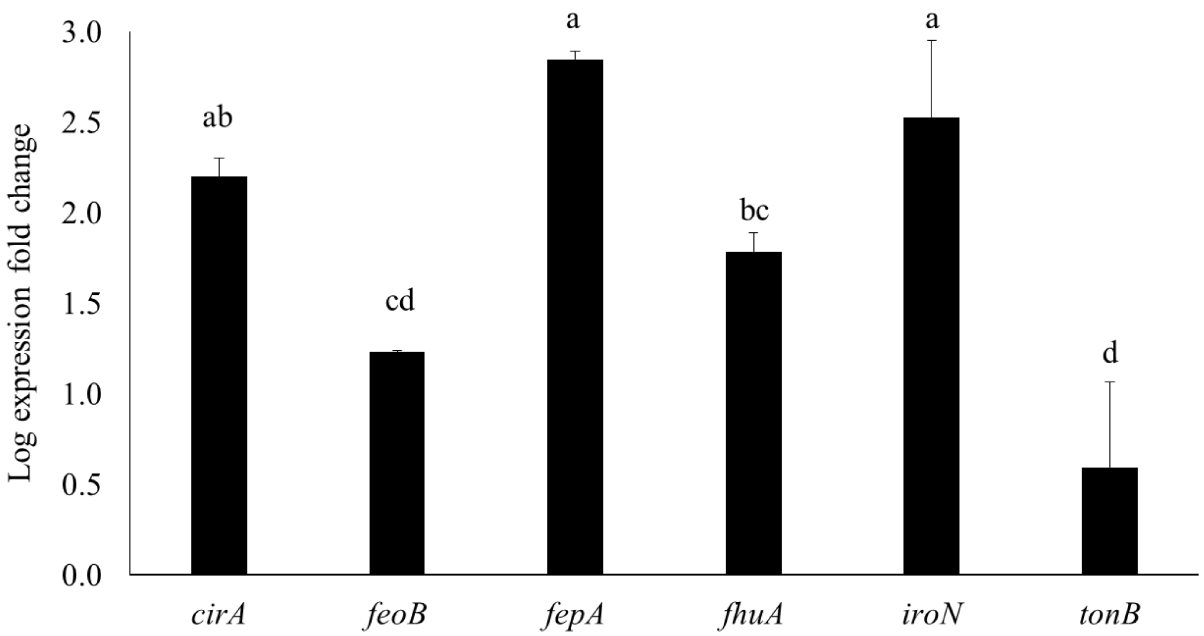

Figure 15. Relative expression of *cirA, feoB, fepA, fhuA, iroN,* and *tonB* in *S.* Typhimurium 3128 WT grown in TS broth with 2,2 dipyridyl incubated at 37°C until mid-log phase growth and compared with *S.* Typhimurium 3128 WT in TS broth at 37°C until mid-log phase growth. Data are means ± standard deviation of three replicates. Means with different letters are statistically different ($P<0.05$).

In TS broth with 2,2 dipyridyl, *S.* Enteritidis 3346 upregulated *fepA, iroN,* and *cirA* more than *feoB* and *tonB* (Figure 17). *FhuA* was upregulated more than *feoB* and *tonB*, although to similar amounts as *cirA*. In UHT milk, *S.* Enteritidis 3346 upregulated *fepA, iroN,* and *cirA*, more than *feoB, FhuA, tonB* (Figure 18).

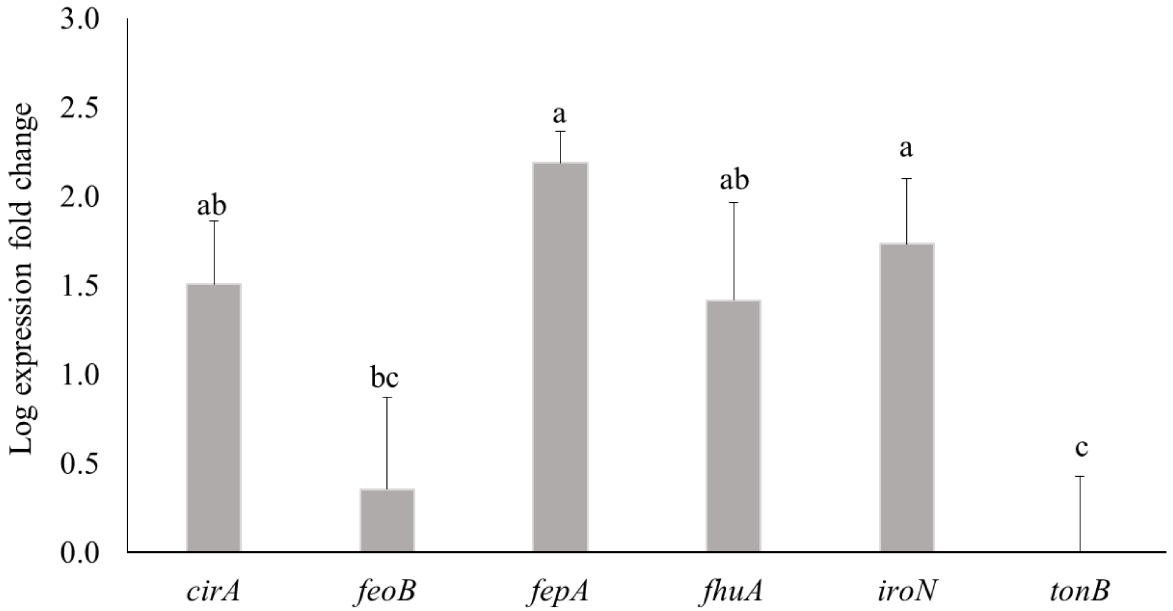

Figure 16. Relative expression *cirA, feoB, fepA, fhuA, iroN,* and *tonB* in *S.* Typhimurium 3128 WT grown in UHT milk incubated at 37°C until mid-log phase growth and compared with *S.* Typhimurium 3128 WT in TS broth at 37°C until mid-log phase growth. Data are means ± standard deviation of three replicates. Means with different letters are statistically different (P<0.05).

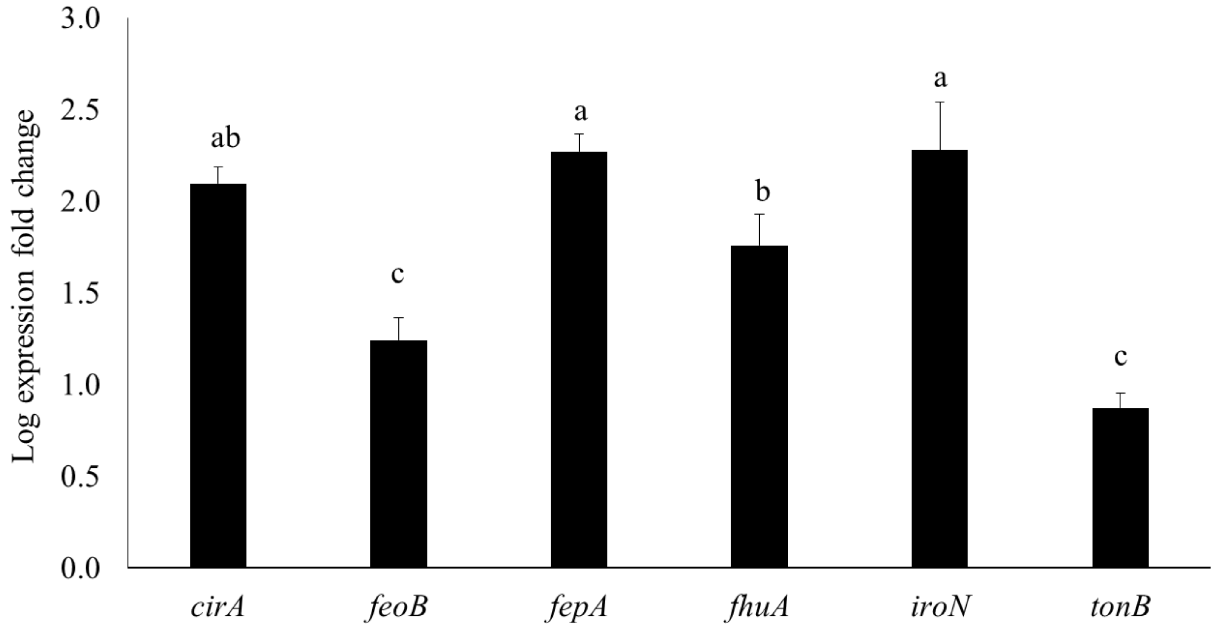

Figure 17. Relative expression of *cirA, feoB, fepA, fhuA, iroN,* and *tonB* in *S.* Enteritidis 3346 WT grown in TS broth with 2,2 dipyridyl incubated at 37°C until mid-log phase growth and compared with *S.* Enteritidis 3346 WT in TS broth at 37°C until mid-log phase growth. Data are means ± standard deviation of three replicates. Means with different letters are statistically different (P<0.05).

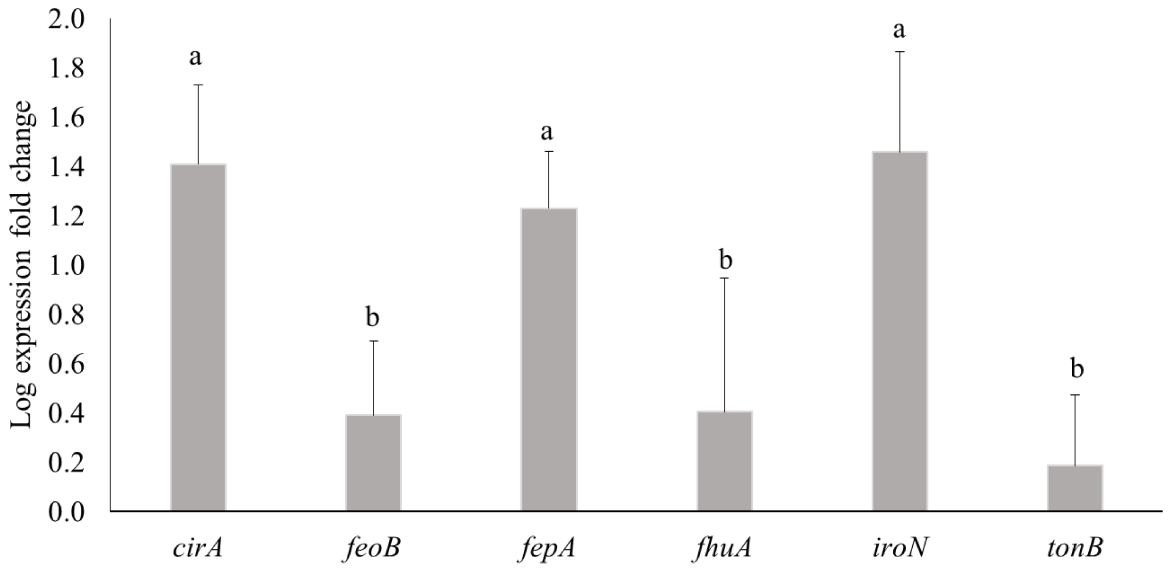

Figure 18. Relative expression of *cirA, feoB*, *fepA*, *fhuA*, *iroN*, *and tonB* in *S*. Enteritidis 3346 WT grown in UHT milk incubated at 37°C until mid-log phase growth and compared with *S*. Enteritidis 3346 WT in TS broth at 37°C until mid-log phase growth. Data are means ± standard deviation of three replicates. Means with different letters are statistically different ($P<0.05$).

Gene expression assays of *S.* Typhimurium 3128 and *S.* Enteritidis 3346 with deletions of *tonB* were also tested. Δ*tonB* mutants grown in both TS broth with 2,2 dipyridyl and in UHT milk were compared with Δ*tonB* mutants grown in TS broth. No differences among the gene expression of the *tonB* mutants and the WT were observed (data not shown).

4. DISCUSSION

4.1 Prevalence of *Salmonella* in lymph nodes

Previous research has established that lymph nodes can harbor *Salmonella* and enter the food chain when muscle and adipose tissue are ground together to produce ground beef (1, 23). One aim of this study was to determine if *Salmonella* was present inside bovine lymph nodes collected from abattoirs in Alberta. It proved very difficult to find a large abattoir that would

allow the excision of lymph nodes from the carcass as it slows their production line. As this study only accepted whole lymph nodes it proved equally as challenging to find discarded adipose tissue with uncut lymph nodes that could be excised and used for detection. The lymph nodes collected came from cattle that were slaughtered as part of other studies and as such there were not a sufficient number of samples available to make solid conclusions about the prevalence of *Salmonella* in lymph nodes of cattle slaughtered in Alberta. None of the 37 lymph nodes tested were positive for *Salmonella* and there was no variation between seasons (Table 7). From this limited evidence, it can be concluded that *Salmonella* is likely present in <2.7% of lymph nodes, as 1/37 would indicate 2.7% positive. To determine an adequate sample size for the population of cattle in Alberta, which is estimated to be 3.34 million head (56), Cochran's formula can be used (57). To obtain a 99% confidence level with 4% margin of error, lymph nodes from 1,034 cattle would need to be analyzed. Although insufficient numbers of samples were taken to prove or disprove the hypothesis, the conclusion supports the hypothesis and is similar to the findings of other researchers (1). More samples need to be obtained in the future to determine the prevalence of *Salmonella* in lymph nodes in Albertan cattle.

To determine if cattle feed was contaminated by *Salmonella* and consequently could be a vector for the pathogen, sixteen bovine feed samples were obtained in duplicate from Lacombe Research Centre. None of the samples contained *Salmonella* (Table 8), although not enough samples were taken to make a conclusion as to the prevalence of *Salmonella* in feed.

4.2 Influence of iron uptake proteins on the growth of *S.* Typhimurium and *S.* Enteritidis in broth, ground beef and UHT milk

Salmonella can uptake ferric and ferrous iron via different systems. Ferrous iron is taken up by FeoB and by the divalent cation uptake proteins, MntH and SitABCD. Ferric iron is only taken up via siderophores. *Salmonella* creates and exports two siderophores, enterobactin and salmochelin. These siderophores can break down into DHBS. IroN uptakes enterobactin, salmochelin, and DHBS. FepA uptakes enterobactin and DHBS, and CirA is only able to uptake DHBS. TonB is necessary to provide energy for all three of the catecholate siderophore uptake proteins, as well as the hydroximate siderophore uptake protein, FhuA. Other researchers have argued that individual iron uptake proteins are crucial for growth and survival (36, 44–47). In these works, different strains of *Salmonella* were studied under vastly different conditions, but each body of research established that iron acquisition is crucial. The aim of this portion of the research was to determine the importance of different iron uptake proteins on the growth and survival of *Salmonella* in broth, ground beef and UHT milk.

Interestingly, *Salmonella* did not grow at 8°C in ground beef (Figures 8 and 11), even though the OD_{600} measurements of *Salmonella* incubated in broth at the same temperature indicated growth (Figures 4 and 7). At temperatures around the growth minimum, *Salmonella* has been known to filament (58). Filamented cells form single colonies when plated on agar, but as the cells grow in length, an increase in OD_{600} is observed. This observation may explain the results observed in this research, as 8°C is close to the minimum growth temperature, the increase in OD_{600} in broth may have been a result of filamentation.

To avoid potential issues with filamentation, differences in growth between the WT and mutants were determined at 12.6°C. No differences were observed between the growth of the WT and mutants of the *Salmonella* strains tested in ground beef, an iron rich environment. In environments where iron is plentiful there is both ferrous and ferric iron available and *Salmonella* can use a ferrous iron uptake system if catecholate siderophore intake is not possible. Δ*tonB* mutants were unable to obtain siderophore mediated iron, as TonB is necessary for siderophore uptake. However, Δ*tonB* mutants grew the same as the WT in ground beef. This indicates that the Δ*tonB* mutants were able to utilize ferrous iron for growth.

In TS broth with 2,2 dipyridyl, the Δ*tonB* mutant of both strains of *Salmonella* were the only mutants that grew to a lesser extent than the others after 4 h incubation (Figure 2 and 5). The iron chelator, 2,2 dipyridyl chelates extracellular and intracellular stores of ferrous iron, but it can also chelate ferric iron (59). Since TonB is responsible for the import of all catecholate siderophores, removing it would mean that *Salmonella* would only be able to intake ferrous iron. By adding 150 mM of 2,2 dipyridyl all the available iron in the TS broth was sequestered. However, populations lacking *tonB* were able to grow, although they grew slower and entered stationary phase after 6 h (Figure 3 and 6). This finding may be due to the rate of iron chelation, both intracellularly and extracellularly. The cells may still be able to use some of their intracellular iron for growth before succumbing to the bacteriostatic effect of iron depletion. The same bacteriostatic effect was observed in UHT milk for *S.* Typhimurium Δ*fhuA-CDB*Δ*iroN*Δ*fepA*, Δ*iroN*Δ*fepA*, and Δ*tonB,* as well as *S.* Enteritidis Δ*tonB* (Figure 10 and 13). The presence of *fhuA-CDB* is unnecessary in these conditions as the growth of Δ*fhuA-CDB*Δ*iroN*Δ*fepA* and Δ*iroN*Δ*fepA* was the same. CirA seems to play no role in iron uptake as there were very minimal differences between *S.* Typhimurium

Δ*iroN*Δ*fepA* and Δ*tonB*, and these differences were only on 2 and 10 d of storage (Figure 10). Since TonB controls siderophore uptake of IroN, FepA and CirA, these findings indicate that CirA is redundant. From this study, the most important iron uptake proteins are FepA and IroN. Knock-outs of either gene did not show the same phenotype as the double mutant, and the WT phenotype was restored when either *fepA* or *iroN* were complemented on a plasmid. Thus, the presence of one of these genes is important for growth, but the presence of both is unnecessary. These findings reinforce previously published data for growth of *Salmonella* in a murine macrophage, also an iron limited environment (45). Either *fepA* or *iroN* were needed to colonize murine macrophages, but not both (45). Survival inside macrophages is necessary for *Salmonella* to eventually end up in ground beef via lymph node contamination, FepA and IroN could be important in preventing this.

The strains with pSCA were grown in TS broth with kanamycin to prevent the loss of the plasmid, the antibiotic possibly effected the growth rate of the initial culture as these strains had lower initial counts in UHT milk.

4.3 Expression of genes related to iron uptake

Pure RNA was very difficult to obtain from UHT milk. During the initial attempts of this experiment, mechanical, chemical and a combination of cell disruption techniques were used in addition to silica-membrane column purification, phenol-chloroform extraction, and a combination of both to determine which method would give the best RNA quality and quantity. In every case the concentrations obtained were less than 11.4 ng/μL. As a result, the same method of extraction of RNA was used for all media. The method used in the current experiments was a published method that had been used for bacterial isolation of RNA from milk

(60). Similar concentrations of DNA were extracted from milk in other research that compared many different kits and methods (61). In future research it would be recommended to add more inoculated UHT milk to the RNAprotect, to increase the amount of RNA in the sample. Then, additional clean up steps could be done without worry of losing what little RNA was able to be purified. However, it is hypothesized that some components of milk may have a negative effect during various aspects of RNA isolation. The lipids in milk may shield the bacteria from chemical lysis, or protect it from mechanical lysis, and the carbohydrates in milk may prevent RNA from binding to the column and are likely a contaminant in the pure RNA eluent, leading to incredibly low A260/230 values that are essentially unreadable. The low concentration of RNA is not a problem for downstream RT-qPCR as there is an amplification step. However, the results obtained from *Salmonella* grown in UHT milk must be interpreted more critically as the quality and integrity of the RNA could not be confirmed due to lack of A260/230 and RIN values. The high variability in the data for growth in UHT milk could be an indication of the poor quality of RNA obtained from these samples (Figure 16 and 18). The results of this experiment confirmed previous findings that iron uptake proteins are upregulated when iron is limited (6, 39, 49, 50). This research added to known findings by showing the extent of upregulation of individual genes. Both strains of *Salmonella* had very similar trends for which genes are upregulated in media with 2,2 dipyridyl and UHT milk. The catecholate uptake genes, *fepA* and *iroN* were generally upregulated most. *feoB*, and *tonB* were upregulated least for both strains and in both medias, *fhuA* was upregulated to similar levels as *cirA* for *S.* Enteritidis and to similar levels as *cirA* and *feoB* for *S.* Typhimurium. This follows with the previous findings that IroN and FepA were crucial for prolonged growth in iron limited environments. Although all iron uptake genes are regulated by Fur and therefor iron limitation causes the upregulation of each of the iron

uptake genes; however, they are not upregulated at the same rate. There are limitations in this work, as only one reference gene was used for the qRT-PCR work, and only one gene in each system was targeted. For a more complete picture of the differences in upregulation, it would be interesting to look at not just one gene from each system, but every gene involved in each system.

4.4 Antimicrobials targeting iron uptake proteins

Colicins are bacteriocins produced by some strains of *E. coli* and when tested against pathogenic strains of *Salmonella* it was found that only group B colicins provided moderate control, exact numbers were not provided; however, it was decided that moderate control was not enough to merit practical use (62). Colicins use a variety of iron uptake proteins to translocate the bacterial membrane in *E. coli*. Group B colicins bind to iron uptake proteins and are translocated using TonB. Microcin J25 is a bacteriocin that targets FhuA, however *S.* Typhimurium and *S.* Enteritidis are resistant to this bacteriocin because FhuA in these *Salmonella* strains do not allow it to translocate as it does through FhuA in *E. coli* (63). Novel research by Schneider et al. (2018) has found a bacteriocin that will inhibit *Salmonella*, which they have named salmocin (62). However, even though these types of bacteriocins bind to TonB, they are not responsible for preventing iron uptake, but are pore-formers, nuclease inhibitors or they prevent peptidoglycan synthesis. If a bacteriocin was found that targeted and prevent IroN and FepA, or TonB activity, it may have potential as an antimicrobial in low iron environments.

The current research leads to another hypothesis, that in low iron environments, using a compound or antimicrobial to prevent catecholate synthesis or uptake would have a bacteriostatic

effect. If true, it would prevent pathogen growth and create a safer food product; although, the food products would be limited to those with very low iron concentrations. More research would need to be completed to determine if there was an antimicrobial that could prevent catecholate siderophore intake or synthesis, as well as determine the range of food products that could be used and the effectiveness and safety of a new food additive.

4.5 Conclusion

In order to test the hypothesis that *Salmonella* is prevalent in lymph nodes of cattle in Alberta, a larger sample size is needed. In future studies, hopefully cooperation of a federally inspected abattoir would make answering this question easier. The prevalence of *Salmonella* in lymph nodes of cattle in Alberta is likely low. The presence of *Salmonella* in lymph nodes is an important issue for food safety and if a high prevalence of *Salmonella* is detected in a larger sample size, this could explain why *Salmonella* is detected in ground beef samples in Alberta.

Catecholate siderophore uptake is important in iron limited environments to prevent the bacteriostatic effect of iron limitation. Due to the redundancy of iron uptake systems individual iron uptake proteins do not cause a bacteriostatic effect in high iron environments. This is important because preventing bacterial iron uptake in meat is not a viable solution for preventing bacterial growth. This strategy may be able to be used in low iron foods, such as milk, if an antimicrobial or compound was found and extensively tested. This work helps determine which iron uptake proteins under specific conditions may be a target for antimicrobials in food products. Since the popularity of raw milk and raw milk cheeses is on the rise, these strategies may be very important for preventing *Salmonella* infection.

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