EFFECTIVE MICROBIAL SUPPLIMENTATION IN THE DIET OF BEEF CATTLE AND ITS EFFECT ON *SALMONELLA* BURDEN AND ITS FOOD SAFETY IMPLICATION: A CASE OF CHERCHER ODA BULTUM UNION FARM

MSc Thesis

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Haramaya, Ethiopia

EFFECTIVE MICROBIAL SUPPLIMENTATION IN THE DIET OF BEEF CATTLE AND ITS EFFECT ON *SALMONELLA* BURDEN AND ITS FOOD SAFETY IMPLICATION: A CASE OF CHERCHER ODA BULTUM UNION FARM

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As Thesis Research advisors, we hereby certify that we have read and evaluated this Thesis prepared, under our guidance, by FUAD MOHAMMED SANI, entitled “**Effective Microbials Supplementation in the Diet of Beef Cattle and its Effect on *Salmonella* Burden and its Food Safety Implication: A Case of Chercher Oda Bultum Union Farm” .** We recommend that it could be submitted as fulfilling the Thesis requirement.

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DEDICATION

This thesis is dedicated to my beloved father Ato Mohammed Sani and my mother W/ro Aliya Shafi for they all have made countless sacrifices for me throughout my life and without whom I would not be the person that I am today.

STATEMENT OF THE AUTHOR

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BIOGRAPHICAL SKETCH

The author, Dr. Fuad Mohammed Sani was born from his mother Aliya Shafi and father Mohammed Sani in West Harerghea zone of Oromia Regional State, Ethiopia on November, 1980. He attended his elementary school at Hirna No-1 junior secondary school and then joined secondary school at Hirna senior secondary school. He joined the then Debub University now Hawassa University, Awassa College of Veterinary Medicine September 2004 and pursued Doctorate Degree in Veterinary Medicine, and graduated in 2009. Thereafter, he worked as an expert veterinarian at woreda and zonal livestock office head in Western Harerghea. He then joined the School of Graduate Studies of Haramaya University in July 2016 for his graduate studies in the field of veterinary public health.

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ABRIVATIONS AND ACRONOMIES

APC	Aerobic plate count
CAPF	A commercial agricultural production facility
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
COBFCU	Chercher Oda Bultum Farmers Cooperatives Union
DFM	Direct Feed Microbial
FDA	U.S. Food and Drug Administration
FSIS	Food Safety Inspection Services
HACCP	Hazard Analysis and Critical Control Point
MDR	Multi drug resistance
MLN	Mesenteric Lymph Node
NARMS	National Antimicrobial Resistance Monitoring System for Enteric Bacteria
PLNs	Peripheral lymph nodes
SLN	Sub iliac Lymph Node
SPS	Sanitary and Phytosanitary
UV	Ultraviolet

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ABSTRACT

Sa*lmonella* remains a persistent public health concern both in the developed and developing countries. Several reports on the prevalence of *Salmonella* in food, food production environment and human cases have been found both in developing and developed countries. A randomized controlled field trial based on parallel group design was conducted from January, 2018 to July, 2018 with the objectives to investigate the preventive effect of supplementing Effective Microbial (EM) in diet of beef cattle in reducing the presence and burden of *Salmonella* in their lymph nodes. The treatment group exposed to feed supplemented by EM and non- exposed (Control) groups was established. The Chercher Oda-Bultum Farmers Union beef Farm was used for the study purpose. The animals were slaughtered after follow up periods of 90, 100 and 115 days at Haremaya University abattoir. Lymphnode samples was collected from which mesenteric LN (n = 130), Subiliac LN (n = 130) and total (n = 260). Isolation and enumeration of *Salmonella* were conducted. Prevalence and load determination analyzed in laboratory total significant reduction (53.00%) relative and (37.00%) absolute risk reduction among control and treatment group (P=0.000) were observed. The significant shift in concentration of *Salmonella* in LNs (MLN and SLNs) due to the influence of supplementation EM (P = 0.000) age in MLN (P = 0.00) and body condition (P=0.025) in SLN, duration of treatment for both have significant interaction (P=0.000). Using EM in the diet of beef cattle is effective in reducing risk of *Salmonella* in both prevalence and burden. Further study in different agro ecology, season, breed, production system should be conducted. Using animal model to investigate the interaction of EM, *Salmonella* and bovine immune system will also be important.

Keywords: Salmonella; Lymphnode; Effective Microbial; Risk reduction

1. INTRODUCTION

1.1. Background and Justifications

Gradual increase in world population and change in lifestyles has resulted in demands for quality oriented foods of animal origin. Meanwhile, the number of incidences of food poisoning cases is increasing throughout the world. In other hand ensuring food safety to protect public health and promote economic development remains a significant challenge in both developing and developed countries. Considerable progress to strengthen food safety systems has been achieved in many countries, highlighting the opportunities to reduce and prevent food-borne disease. However, unacceptable rates of food borne illness still remain and new hazards continue to enter the food supply (FAO, 2006). In this regard many emerging and re-emerging pathogens those associated with fresh or raw meat can be mentioned including *Salmonella.*

Fresh meat is highly prone to contamination regardless of its nutritional values. In mild to severe illness, hospitalization or even death can be caused due to ingestion of contaminated food (Ayehu *et al.*, 2014). In Ethiopia, like other developing countries, it is difficult to evaluate the burden of food-borne pathogens. These are because of the limited scope of studies and lack of coordinated epidemiological surveillance systems. In addition, under-reporting of cases and the presence of other diseases considered to be of high priority may have overshadowed the problem of food-borne pathogens (Oosterom, 1991; Edget *et al.*, 2014). The widespread habit of raw beef consumption is a possible potential cause for the spread of food-borne illnesses in Ethiopia (Edget *et al*., 2014).

On the other hand, even though there is scarcity or no precise data, the incidence of food -borne outbreaks in Ethiopia seems to be higher compared to developed countries (Tavakoli *et al.*, 2008). A few studies conducted in different parts of the country showed that pathogenic organisms like *Campylobacter Spp, Salmonella*s *Spp, Taenia Spp, Toxoplasma Spp, Mycobacterium Spp, Brucella Spp, Escherichia coli, Echinococcos/hydatid* cysts were identified as causes of food-borne illness (Zelalem *et al.*, 2015; Bean *et al,* 1990; Tesfay *et al.*, 2014). These and related issues rises the necessity of establishing important food safety measures.

Sa*lmonella* remains a persistent public health concern both in the developed and developing countries. The majority of non-typhoidal salmonellosis cases are associated with food borne vehicles including beef. Even if the implementation of pathogen reduction plans based on the principles of HACCP in the mid-1990s, the contamination of the surface of carcasses with *Salmonella* has declined, but there is no significant reduction in ground beef contamination by *Salmonella*. Moreover, the incidence of human disease has not meaningfully declined over time despite concerted efforts to affect change (FAO, 2010). Current estimates indicate that exposure to non- typhoidal *Salmonella* results in 93.76 million GIT illnesses and 155,000 deaths worldwide each year (FAO, 2016).

In Ethiopia some studies have been conducted in different chain of productions like environmental, abattoir lines, processing lines and animals itself including lymph nodes. The prevalence of 26.6%, 23.5%, and 8.8% has been reported in abattoir line, animals' feces and lymph nodes respectively. Positive results from the lymph nodes indicate the infection status of the animals. Positive environmental samples ranged between 30.7% in knives and 60% in refrigerators. The same study reported 8.3%, 45.5% and 32.4% prevalence from cleaning water, meat transporting track and raw beef from butcheries respectively (Hiko *et al.,* 2016). Approximately the same rates were reported in the same or related chain of beef in Ethiopia (Nyeleti *et al*. 2000; Teklu and Negussie, 2011; Sibhat *et al.*, 2009).

The above ground implies that the ubiquitous nature of *Salmonella* and its prevalence in beef chain in the country. The isolation of related or similar serotyps from both human and animals reveals its zoonotic and food safety implication in the country. In spite of real increments in *Salmonella* prevalence from abattoir to refrigerators no one of the above studies consider the presence of LNs as risk factor.

To reduce the public health risk, clearly more needs to be done in *Salmonella* prevention. Therefore, the purpose of this study was to provide prevention and control options for *Salmonella* carriage in cattle and potential risk for people through reducing the *Salmonella* burden from bovine lymph node. The Effective Microbial (EM) supplement to the diet of beef cattle was used as a means of intervention, to reduce the pathogen in ground beef.

1.2. Statement of the Pproblem

Non-typhoidal *Salmonella* is a leading cause of death due to bacterial contamination of food In USA (Scallan *et al.*, 2011). *Salmonella enterica* has been frequently recovered from the hides and feces of healthy cattle (Callaway *et al.*, 2005; CDC, 2002), and it is theorized that animal carriage of *Salmonella* ultimately contributes to ground beef contamination (Bacon *et al.,* 2002). However, despite successful control of surface contamination, it is still possible for *Salmonella* to be recovered from ground beef as well as the human burden of illness caused by this pathogen has persisted over time (CDC, 2011). In a study of commercial ground beef from seven regions of the U.S. (n=4,136 samples collected over two years), *Salmonella* was recovered from 4.2% of ground beef samples (Bacon *et al.,* 2002). In some regions, cattle appear to be a natural reservoir for *Salmonella* (Kunze *et al.,* 2008; Rivera-Betancourt *et al.,* 2004)*;* as a consequence, beef products are at risk of contamination. However, estimates of the prevalence of *Salmonella* among feedlot cattle and those cattle presented at abattoirs are quite variable (Barkocy-Gallagher *et al.,* 2003). This implies feedlot is important area to be searched. According to Sibhat *et al.* (2009) and Hiko *et al.* (2016) in Ethiopia, *Salmonella* preventive issues were raised.

The primary source of *Salmonella* for cattle occurs at the farm level. Therefore, on-farm control of *Salmonella* may assist in reducing *Salmonella* carriage and potential transmission during cattle production, thereby minimizing the *Salmonella* contamination of cattle destined for beef processing. On-farm control of *Salmonella* may thus contribute to the whole food chain continuum of measures for reducing the food safety risk of *Salmonella* in beef (FAO, 2016).

Research suggests that pathogen contamination of ground beef also can occur by means of contaminated lymph nodes (LNs). Cattle possess many LNs located within fatty tissues that are frequently incorporated into ground beef and thus have the potential to contaminate final product. Contaminated LNs may explain the difference in *Salmonella* prevalence between post-intervention carcasses or trim, and ground beef. When present in LNs, *Salmonella* are protected from chemical and thermal antimicrobial carcass interventions, and as a consequence sanitary harvest procedures may not address this potential source of contamination (Arthur *et al.,* 2008)

A recent research finding reports that there is significant reduction in *Salmonella* prevalence in Sub iliac Lymph Node (SLNs) of cattle administered NP51 and NP24; however the effect varied across slaughter days and regions across USA and the world. A relative risk reduction of 50% and 31% was observed in *Salmonella* prevalence on the first and second slaughter dates, respectively. Finally, the authors recommended that the efficacy of direct feed microbial to the diet of beef cattle should be evaluated in different parts of the world (Vipham *et al.*, 2015). The analogous product being used in Ethiopia is EM2, which includes *Lactobacilus spp* as a component. Although currently EM is used for production and productivity purpose the diverse use of this technology is not yet well studied and exploited. The application of this technology prevents disease development and epidemics **t**hrough the principle called competitive exclusion; EM can limit the population of pathogenic microbes like *salmonella, Enterococci* and *E. coli* thus suppressing their pathogenic activities. Aside from that, EM improves the general health of the animals thus increasing the resistance to diseases (Edens *et al.* 1997; Kitaw *et al.*, 2007).

Ethiopian scenario risk factors for carcass and ground beef contamination by pathogenic *Salmonella* were reported as hide, rumen contents, abattoir facilities, food handlers, storage facility, transportations and processing and accordingly recommended different sanitary and hygienic measures in post harvest chain (Ejeta *et al*., 2004; Anbessa *et al.,* 2012 ; Endale *et al.*, 2013, Hiko *et al.*, 2016). However, the potential of biotechnological application in *Salmonella* prevention/reducing the burden in beef at pre-harvest stage was not yet conducted using effective microbial (EM) inoculants biotechnology.

1.3. Research Qquestions

- Does the Effective Microorganisms (EM) supplementation in diet of beef cattle significantly reduce the presence and burden of *Salmonella* in Sub- iliac lymph and Mesenteric lymph nodes of cattle?
- Does supplementation of EM in diet of beef cattle prevents the presence and burden of *Salmonella* in Sub- iliac lymph node which potentially incorporated to ground beef?

1.4. Significance of the Research

The livestock production system in Western Hararghe is market oriented. Fattening is commonly practiced by all farmers in all places. In this area, the crop production system in all agro-ecologies gives priority to livestock. All farmers, irrespective of the agro-ecology, grow sorghum and maize mainly targeting animal feed. Crop production is secondary. These crops are selected for their high biomass as animal feed. The demand for fattening even shaped the system of cropping as fattening package of Hararghe farmers is based on sorghum and maize leaves, seedlings, tassels and defoliated leaves. Besides, the study area is nationally known fattening belt which supplies Addis Abeba, Adama, Mojo and Bishoftu abattoirs in domestic and live animals export market to the Middle East (Gebresgaber and Gebrehiwot, 2011).

The Chercher Odabultum Farmers Union (COBFCU) is market out let of beef cattle for member cooperatives of eight districts. Currently, the farm doesn't have a pre-harvest stage *Salmonella* prevention measure as a tool for good hygienic production. Thus, the application of EM technology and having the finding as farm protocol promotes its competency in market by developing reliability among its customers by supplying quality, quantity and safe beef. This increases the profit margin of the farm which will be shared by member of cooperatives through dividend which directly promotes household income of the members. The participation of COBFCU in this study facilitates the knowledge management process through its regular capacity building programs for its cooperative members. Generally, this study will develop pre-harvest intervention option for the prevention and control of *Salmonella* in beef and strengthen the HACCP of the farm from which the findings will be applied at national level.

1.5. Objective of the Research

- To assess and evaluate the effect of applying EM-supplement in the feed of beef cattle on the presence and burden of *Salmonella* in their Mesenteric and Sub iliac Lymph Nodes.
- To investigate the preventive efficacy of supplementing EM technology in the diet of beef cattle in the presence and burden of *Salmonella* in their Mesenteric and Sub iliac Lymph Nodes.

2. REVIEW OF LITRATURES

2.1. General Ooverviews of *Salmonella*

The non-typhoid *Salmonella* serotypes, which include some 2500 different serotypes, are widely distributed in nature, including in the gastrointestinal tracts of mammals, reptiles, birds, and insects. Most clinical infections of humans are transmitted from healthy carrier animals to humans through food. The main clinical manifestation of human infection with non-typhoid *Salmonella* is an acute gastrointestinal illness and, less frequently, septicemia. Non-typhoid *Salmonella* was among the earliest of the so-called emerging pathogens reviewed in (Riemann and Cliver, 2006).

The development of serotyping was fundamental for the understanding of the epidemiology of *Salmonella* infections. The foundation for this serotyping scheme was the discovery of the flagellar H antigen and the thermostable somatic O antigen by Weil and Felix and the phase-shift in the H antigen. The genus *Salmonella* belongs to the family *Enterobacteriaceae, which* consists of Gram-negative, facultative anaerobic, nonspore- forming rods (Riemann and Cliver, 2006).

In addition to these conventional methods molecular methods are also available. Separation of large restriction fragments, generated by restriction enzymes with few recognition sites in the chromosome, by pulsed field gel electrophoresis (PFGE) is currently the molecular 'gold standard' for *Salmonella* typing, and good standardized protocols have been developed for use with *Salmonella* (Swaminathan *et al*., 2001). PFGE profiling effectively subdivides most serotypes, where other methods fail to do so as reviewed in (Riemann and Cliver, 2006).

2.1.1. Virulence Mmechanisms and its Genetic base of *Salmonella*

More than 4 % of the genetic information in *S*. Typhimurium has been associated with fatal disease in a mouse model, indicating that *Salmonella* are highly specialized pathogens (Bowe *et al*., 1998). The virulence genes are scattered throughout the chromosome and in some serotypes on large virulence-associated plasmids. Large genetic elements, termed *Salmonella* pathogenicity islands (SPIs), are essential for virulence (Zhang *et al*., 2003).

The SPI1-genes encode a type-three secretion system, which forms a needle complex. It secretes effector molecules into the membrane and cytosol of the host cell (reviewed by Zhang *et al.*, 2003). Studies suggesting that neutrophil-induced damage or signaling is significant in development of diarrhea. The inflammation leading to necrosis of the intestinal mucosa has also been implicated as an important cause of fluid and protein loss during *Salmonella* infection (Santos *et al.*, 2002). It has been suggested that the inflammation is not just caused by the invasion of the bacterium in the intestine, but also by induction of signaling molecules (Zhang *et al.*, 2003).

Salmonella that cause systemic infection are taken up by phagocytic cells in the lamina propria. From there they are transported to systemic sites by exploiting the ‘normal’ cell trafficking system. It has been suggested that macrophages are mainly responsible for the transport of bacteria, but peripheral dendritic cells also contain *Salmonella* during the infection and may help *Salmonella* to reach the draining lymph node (Yrlid *et al.*, 2001). Genes encoded in *Salmonella*-pathogenicity island 2 (SPI2) are essential for systemic salmonellosis in animal models (Jones *et al.*, 2001). SPI2, which is absent in *S. bongori*, encodes a type-three secretion system that translocates effector molecules into the cytosol of the infected cell. The effector molecules are not well characterized (Hensel, 2000), but are known to affect phagosome–lysosome formation and cause exclusion of NADPH oxidase from the *Salmonella*-containing vacuole (Vazquez-Torres *et al.*, 2000).

2.1.2. Host Susceptibility Resistance and Immunity in Animals

Salmonella are generally described as being virulent microorganisms whether they illicit illness or exist in the carrier state. Many factors may contribute to the type and degree illness those results from infection, including: host susceptibility, virulence of the *Salmonella* strain, infectious dose and route of infection (Janda and Abbott, 2006). During the infection process, *Salmonella* must adapt to changes within the host. *Salmonella* must delicately orchestrate the action of many gene products in the proper location at the proper time to ensure survival within a host (Marcus *et al.*, 2000). *Salmonella* invade through the damaged tissue and gain access to underlying tissues to penetrate the intestinal epithelium. Following invasion, *Salmonella* infects phagocytes (Monack *et al.,* 2004), (i.e. macrophages and dendritic cells) and these phagocyte-encased cells enter the lymphatic system, where they are drained to the mesenteric lymph nodes (Jones and Falkow, 1996).

2.1.3. Common Reservoirs and Modes of Transmissions

Cattle, swine, and poultry are known to harbor and shed *Salmonella* capable of causing disease in humans; thus, these species are considered to be important reservoirs for this pathogen. The attribution of human *Salmonella* infections from food-producing animals has been described previously (Doyle, 2013). Furthermore, it has been shown that the classification of *Salmonella* serotypes among animal reservoirs has proven to be informative as some serotypes are associated with different reservoirs and, therefore, may have differing vehicles for human exposure (Greig and Ravel. 2009). For example, *Salmonella* serotypes commonly associated with cattle include: *S*. serotype Anatum, *S*. Montevideo, *S*. Dublin, and *S*. Infantis (Doyle, 2013). Interestingly, however, the serotypes recovered from ground beef differ, in that *S*. Montevideo, *S*. Dublin, *S*. serotype Cerro, *S*. Newport, *S*. Anatum, *S*. serotype Muenster, and *S*. Mbdanka are the most prevalent (Doyle, 2013). Alternatively, the same serotypes associated with chickens are commonly found in ground chicken, namely *S*. Kentucky, *S*. Enteritidis, *S*. Heidelberg, *S*. serotype I 4,5,12:i:-, and *S*. Typhimurium (Doyle, 2013). Attribution data provided for reservoirs and food vehicles associated with each *Salmonella* serotype are valuable to inform future research, risk management, and aid in the development of pathogen inhibition in the food production chain to limit human illness (Pires *et al.,* 2009).

2.1.4. Important Serotypes of Zoonotic *Salmonella* and Public Health

More than 2,500 *Salmonella* serotypes are recognized to date. Many are known to cause illness in humans, yet the majority of human illnesses are attributed to a relatively few serotypes. *Salmonella* serotypes vary considerably in terms of invasiveness and rates of illness. Various serotypes have been associated with causing mild to severe illness, depending on virulence factors and the immune status of the individual. Current research shows that a select few serotypes can cause severe illness in relatively few infected persons (e.g., *Salmonella* serotype Dublin and *Salmonella* serotype Choleraesuis), while others (e.g., *S*. Typhimurium, *S*. Enteritidis, and *S*. Newport) are responsible for a larger proportion of the total salmonellosis cases (Jones *et al.*, 2008)

Salmonella Typhimurium is the second most prevalent serotype isolated from food, accounting for 14% of laboratory-confirmed cases of salmonellosis (Crim *et al.*, 20014). *Salmonella* Typhimurium is also one of the top serotypes isolated from food-producing animals and retail meats (Louden *et al.,* 2012). In a six-year span from 2007 to 2013, 61 outbreaks of *S*. Typhimurium were recorded for animal contact (e.g., frogs, hedgehogs, and turtles) and a variety of food sources such as beef, cantaloupe, lettuce, chicken, and eggs (CDC, 2014).

2.2. Epidemiology of *Salmonella* in Pre- Harvest Chain of Beef

It has been well documented that ruminants make excellent hosts for *Salmonella* and thus it can be easily disseminated in the feces (Callaway *et al.*, 2010; Kunze, *et al.*, 2008). *Salmonella* are pathogens capable of residing as transient members of the intestinal microbial population within bovine species (Callaway *et al.*, 2008). Although the prevalence of *Salmonella* is high in US, especially in the southern region (Blau *et al.*, 2005; Dodd *et al.*, 2011; Kunze *et al.*, 2008), the incidence of salmonellosis does not reflect this in mature cattle (Cummings *et al.,* 2009; Edrington *et al.*, 2008). Young animals are frequently colonized by *Salmonella* and are most likely to experience salmonellosis within 2-4 weeks of age (House *et al.*, 2001). A large proportion of mature cattle in the south are infected, but show no clinical signs of *Salmonella* infection leading to a high number of asymptomatic carriers (Dodd *et al.*, 2011). Thus, reliance on overt clinical indicators of illness is not an effective indicator of *Salmonella* colonization, as infected animals may appear healthy (Callaway *et al.*, 2008). Reasons behind the absence of clinical signs of *Salmonella* infections in cattle are currently uncertain. However, recent data has shown that rather than acquiring this pathogen after birth, animals may be infected in utero (Heithoff *et al.*, 2012).

In Ethiopia studies show that there is significant burden in beef chain. The prevalence of *Salmonella* from minced beef in Addis Ababa reported by Ejeta *et al.* (2004) was (14.4%). In other studies 40% and 42% prevalence was also reported from minced meat (locally known as «kitfo») while the samples were collected from different hotels, bars and restaurants in Addis Ababa from the same area (Molla *et al.*, 2000; Tegegne and Ashenafi, 1998). According to data compiled by Edget *et al.* (2014), through systematic review 15 studies were published on food born *Salmonella* pathogen from 2000 – 2013 most of them were concentrated in central part of Ethiopia. Thus this makes difficult to describe its epidemiology in terms of national figure. However, this piece of information should guide us to the pre-harvest chain to reduce this burden.

2.2.1. *Salmonella* in Bovine Lymph Node

2.2.1.1. Its food safety implication

A number of outbreaks and recalls have been reported as a result of *Salmonella*-contaminated ground beef products (CDC, 2013; McLaughlin *et al.,* 2006). Research has suggested that the carriage of such pathogens by cattle may contribute to the overall prevalence of contaminated ground beef products. Despite the apparent success of surface decontamination intervention efforts, surveillance measures have estimated that the prevalence of *Salmonella* in ground beef products may range between 2.0 and 4.2% (FDA, 2011). As a result of this ongoing food safety concern, the beef industry is investigating alternative routes of contamination with the anticipation of developing a strategy to mitigate the burden of *Salmonella* in ground beef. Consequently, recent publications have provided evidence that pathogen contamination of ground beef products may also occur via the animal's lymphatic system, specifically through the inclusion of PLNs in ground beef products (Arthur *et al.,* 2008; Gragg *et al.,* 2013; Haneklaus *et al.,* 2012). About 14% of the slaughtered cattle carried *Salmonella* in caecal contents and/or mesenteric lymph nodes (Sibhat *et al.,* 2011). The 8.8% from MLNs was also reported from apparently healthy animals (Hiko *et al.,* 2015). Both authors suggested that the detection of *Salmonella* in caecal contents and mesenteric lymph nodes of slaughtered cattle is of significance in food safety as this can easily result in contamination of carcasses and edible organs.

2.2.1.2. Bovine lymph node as epidemiological niche

Importantly, it has been noted that *Salmonella* can be recovered from various PLNs that are distributed throughout the carcass. While *Salmonella* has been recovered from lymph nodes of differing anatomical origins, widespread dissemination of *Salmonella* throughout the lymphatic system does not appear to be common (Gragg *et al.,* 2013).

Many early lymph node studies focused their efforts on investigating *Salmonella* in the mesenteric lymph nodes (Arthur *et al.,* 2008; Samuel *et al.,* 1981; Sofos *et al.,* 1999); however, it is noteworthy that gastrointestinal tract (GIT)-associated lymph nodes, such as these, are discarded during the evisceration process and, thus, do not pose a direct food safety hazard. In contrast, PLNs that reside in the adipose tissues are associated with important muscle cuts; it is these PLNs that should be used to determine the magnitude of the food safety hazard posed by harborage of *Salmonella* in the PLNs of healthy cattle presented for harvest, as they have greater potential to be incorporated into ground beef products (Arthur *et al.,* 2008). Indeed, a recent risk assessment albeit limited by available data and parameter estimates indicated that the contribution of *Salmonella* in ground beef is largely from PLNs compared to *Salmonella* from the carcass surface (Li *et al.,* 2014). Due to the complexity in removing certain lymph nodes at harvest, many recent studies have focused on examining PLNs that are more accessible during harvest and may be important in regards to food safety, including the popliteal, pre-scapular (chuck), and Subiliac (flank) (Arthur *et al.,* 2008; Gragg *et al.,* 2013). As exploration of *Salmonella* in the PLNs is in the early stages, publications and data are relatively scarce regarding epidemiological trends associated with harborage. Preliminary research suggests that prevalence of *Salmonella* in PLNs of healthy cattle presented for harvest can range between 1.6 and 88% (Arthur *et al.,* 2008; Gragg *et al.,* 2013; Gragg *et al.,* 2013; Haneklaus *et al.*, 2012).

In an exploratory study by Gragg *et al.* (2013) an overall mean prevalence of 7.5% was observed in 3,327 Subiliac lymph nodes. Importantly, the authors reported trends suggesting that harborage of *Salmonella* may be affected by factors such as animal-type (i.e. feedlot and cull animals), season, and region; moreover, the authors reported that the overall mean prevalence may have been skewed by these variables. Upon stratification of the data, it was observed that the prevalence of *Salmonella* was greater in the feedlot cattle populations relative to cull cattle populations (Gragg *et al.* 2013). *Salmonella* prevalence in the cull cattle populations remained consistently low (0.65%) and did not appear to be affected by region or season. Alternatively, *Salmonella* prevalence in the feedlot cattle populations appeared to be low in the cooler season yet peaked in the warmer season, particularly in the southwest region of the US. An additional study evaluating *Salmonella* prevalence in lymph nodes collected from cattle presented for harvest in Mexico supports the findings of a seasonal and regional trend (Gragg *et al.*, 2013). As previously discussed, similar trends have been observed in fecal, hide, environmental, and food sample data (Barkocy-Gallagher *et al.*, 2003; Edrington *et al.*, 2004). Alternative influential variables have been hypothesized including animal temperament, animal stress levels, management styles, feeding regiments, animal origins, and environmental factors (Gragg *et al.* 2013; Haneklaus *et al.,* 2012). In addition to the seasonal and regional trends, Haneklaus *et al.* (2012) demonstrated that *Salmonella* prevalence in PLNs may also vary among feedlot facilities within the same geographic region. In this study, 307 pre-scapular and subiliac PLNs were collected from seven different feedlot facilities over a three-month span. Importantly, it was noted that during each sample collection, all PLNs from one facility tested negative for *Salmonella*, while PLNs from another facility tested positive for *Salmonella* with prevalence ranging from 76 to 100% (Haneklaus *et al.,* 2012). Despite a relatively small sample size, these findings may illustrate that variables at a feedlot level, such as animal husbandry practices, animal origin, and environmental factors, may greatly influence *Salmonella* harborage in the PLNs.

Also noteworthy is the characterization of the *Salmonella* isolates collected from PLNs, as potential risks associated with product contaminated by particular *Salmonella* serotypes harboring virulence factors or antimicrobial resistance exist. Various *Salmonella* serotypes have been recovered from PLNs, with some publications reporting as many as 24 different serotypes (Gragg *et al.,* 2013). While some diversity has been observed, a majority of the isolates reported by Gragg *et al.* (2013) were *S*. Montevideo and *S*. Anatum (44% and 25%, respectively), which have also been reported as the two most commonly isolated serotypes in ground beef products (Anderson *et al.,* 2001; FSIS, 2011). *S*. Typhimurium and *S*. Newport, which are commonly associated with human illness, have been recovered from PLNs; while the prevalence of these serotypes was reported to be relatively low, it is noteworthy that these were largely isolated from cull animal populations (Gragg *et al.*, 2013). While, overall, antimicrobial susceptibility testing revealed that a majority (86%) of *Salmonella* isolates collected from PLNs were pan-susceptible, 8.3% were MDR, which (as opposed to the discussion above) was defined as resistance to two or more antimicrobial classes (Gragg *et al.*, 2013). Notably, MDR phenotypes were also more commonly isolated from cull cattle PLNs relative to feedlot cattle PLNs. This may indicate that while *Salmonella* prevalence in cull animal populations remains relatively low throughout all regions and seasons, the presence of medically important serotypes and MDR strains within this population may warrant further investigation to decrease the potential risk imposed.

2.2.1.3. Root of entry to lymphatic system

The growing recognition of *Salmonella* in PLNs has also generated questions regarding the route by which *Salmonella* infects the PLNs and the duration of infection (Edrington *et al.,* 2013a; Edrington *et al.,* 2013b; Gragg *et al.,* 2013). Upon entry into the body, an intruder, such as a virus or bacteria, is recognized and engulfed by the cells of the immune system to be transported to the lymph node for destruction. The lymphatic system works in branches, with various parts of the body draining to specific lymph nodes within relatively close anatomical regions. The SALNs for example, is responsible for the filtration of lymph draining from the skin of the abdominal wall, pelvis region, and hind limbs (Edrington *et al.*, 2013a; Edrington *et al.,* 2013b; Gragg *et al.*, 2013). Experimental models have been performed, in which cattle were challenged with *Salmonella* via hypothesized routes of infection, namely oral, subcutaneous injections, and intradermal injections, with the objective of achieving PLNs that are predictably *Salmonella*-positive. As *Salmonella* is typically associated with the GIT, initial hypotheses proposed that *Salmonella* in the PLNs might originate from the GIT. Exploratory challenge models conducted by Edrington *et al.,* (2013) demonstrated that *Salmonella* can reach the PLNs via oral exposure. While natural oral inoculation is not an impractical route of infection, the observations suggested that the concentrations of oral exposure necessary to achieve PLNs that are predictably *Salmonella*-positive at a detectable level are substantial and may not be typical of naturally occurring environmental settings (Edrington *et al.*, 2013).

Consequently, alternative hypothesized routes of infection have been investigated in which *Salmonella* infection of the PLNs occurs via transdermal routes, namely insect bites or abrasions on the hide, and is then drained to the regional PLNs (Edrington *et al.,* 2013; Gragg *et al.,* 2013). As a result, a transdermal challenge model was developed in which various *Salmonella* serotypes were applied to the skin of the animal using a multi-prong inoculator allergy skin-testing device that allowed for greater control of penetration depth during application (Edrington *et al.,* 2013a; Edrington *et al.,* 2013b). This method was also able to yield predictably positive PLNs in the corresponding region of the animal that was inoculated with *Salmonella* without resulting in swelling and lameness. While it was reported that this device produces PLNs with *Salmonella* concentrations above the limit of detection for at least eight days post-inoculation, a salient limitation of this approach is that these concentrations were below the level of quantification (Edrington *et al.,* 2013)

A recent publication identified biting flies, such as horned flies (*Haemetobia irritans*), as an opportune route of entry for *Salmonella* to breach the skin barrier, thus resulting in drainage into the regional lymph node as part of the animal's immune response (Olafson *et al.,* 2014). It has been illustrated that *Salmonella* harborage can occur in the fly's mouthparts and digestive tracts, a contamination that may transpire through grooming practices or while pursuing fresh fecal pats for egg deposition. As previously discussed, it is through such events that flies, w hen feeding, may mechanically transmit the bacteria from the animal's hide or environment into lesions created in the skin barrier (Olafson *et al.,* 2014).

2.2.1.4. The lymphatic system and payer's patches

Peyer's patches are associated with the intestinal epithelium and play a large role in intestinal immunity. Microfold cell (M-cells) is a part of the intestinal epithelial and sit directly above Peyer's patches (Parham, 2009). These cells are unique from other epithelial cells due to a specialization in phagocytosis and transcytosis across the epithelium. This is due in part to their particular morphology that allows for more movement of macromolecules, antigens, and commensal and pathogenic microorganisms across their membrane (Mabbott *et al.,* 2013).

Contained within the Peyer's patches are a population of immune cells that include mononuclear phagocytes and naïve and memory lymphocytes. Part of the function of the Peyer's patch and M-cell permeability is to allow secretory IgA produced by resident B-cells to migrate across the epithelial border. However, the function of the Peyer's patch does not stop there. Dendritic cells within Peyer's patches play a role in pathogen phagocytosis and processing into peptides for T-cell presentation. However, dendritic cells can also traffic pathogens to the draining lymph and carry the pathogen to mesenteric lymph nodes (Mabbott *et al.,* 2013).

2.3. Overview of Effective Microorganism Technology

Effective Microorganisms (EM) were developed by the Japanese horticulturalist Professor Dr. T. Higa of the University of the Ryukyus in Japan. EM is a solution containing over 80 species of co-existing microorganisms selected from thousands of microbial species. Microbial species commonly utilized in the food and fermentation industries were selected and included in EM technology (Higa and Wididana, 2007). According to Higa (1994), EM was developed from three principal microorganisms; namely photosynthetic bacteria, lactic acid bacteria and yeast. These were subsequently enriched by other species such as filaments, fungi and *Actinomycetes* at a pH of below 3.5.

The concept of EM is based on the inoculation of mixed cultures of beneficial microorganisms in to the system where they shift the microbiological equilibrium. A series of inoculations were made to ensure that the introduced microorganisms continue their dominance over the indigenous populations (Higa, 1994). The exact mechanism of how EM act and interact in the microbial solution is not known. However, the suggested microbial course of actions within the EM solution include, suppression of pathogens and disease-causing microorganisms, conservation of energy, solubilization of minerals, microbial-ecological balance, increased a source of energy for other microbes, and biological nitrification. The microorganisms coexist and act synergistically when applied. They decompose organic compounds and produce various low-molecular organic compounds, such as amino acids, sugars, vitamins, enzymes and other bioactive substance (Higa and Wididana, 2007).

According to Higa and Wididana (2007) EM represent two dynamic and opposing forces (forces of degeneration and regeneration) existing within nature, since EM are a mixture of regenerative microorganisms naturally adapted to consume degenerative ones. EM was first developed to make the agricultural sector free from the side effect of large scale commercial fertilizer. The use of EM was further expanding to overcome environmental issues and currently, the role of EM in facilitating the re-use of most wastes and environmental management is of significant importance (Higa and Wididana, 2007).The first concept of using EM in environmental management were initiated in the process of composting crop residues and animal wastes aimed at producing bio-fertilizer (Shintani *et al.,* 2000). EM was also effectively used in purification and re-use of urban water sewerage for the use of garden and toilets. The Biological Oxygen Demand and Chemical Oxygen Demand in urban water were found to be reduced when treated with EM (Okuda and Higa, 1999). Some of the benefits claimed to accrue from the use of EM include improved meat and egg quality, improved animal health status, reduction of foul smells and absence of toxic effects on animal farms (Safalaoh, 2006).

In Brazil EM were included in sheep feed and sheep that were fed EM-treated silage consumed greater amounts of silage per unit of body weight compared with untreated silage, which also improved in situ degradability of elephant grass silages (Guim *et al.,* 1999). Integrate livestock and poultry farms in South Africa (Hanekon *et al.,* 2001; Safalaoh and Smith, 2001) and swine and fish farms in Australia widely used EM to increase productivity. The improvement in the production performance of animals receiving EM is said to be attributed to the better-feed conversion efficiencies achieved as a result of inclusion of EM (Konoplya and Higa, 2000; Safalaoh and Smith, 2001).

2.4. Effective Microorganisms in Animal Production and Health

Effective microorganisms (EM) are a naturally fermented liquid probotic utilizing effective microorganisms, a technology developed in the early 1980s in Japan. The basic groups of microorganisms in EM are lactic acid bacteria (commonly found in yogurt, cheeses), yeast (bread, beer), and phototrophic bacteria. The microbes in EM are non-harmful, non-pathogenic, not-genetically-engineered or modified, and not-chemically-synthesized (Gaetane and Dylan, 2008).

EM technology is far superior to regular robotics, which are typically lactic-acid bacteria. Rather, EM comprises an entire consortium of the exact same microbes that are found in natural soils, closely mirroring the microbes that animals should be receiving naturally through their foods (Jason, 2012).

In livestock farming, EM are used for animal health, increased feed conversion, handling of mastitis, handling hoof infection, increased quality of milk or meat and reduction of odors and pathogens. It is easy to implement, and to get better results in a relatively short period of time. EM technology is also highly cost-effective and will actually increase income and profits rather than act as being an added expense (Jason, 2012).

Through worldwide scientific research it is becoming increasingly evident that a major portion of illnesses or ailments in all living organisms are tied to imbalances in bacteria. Today there is more than sufficient evidence to suggest that the long-term health of living organisms is completely dependent on the bacterial health of that organism, and that almost any disease or lowered quality/quantity of production can be resolved through the use of beneficial bacteria. The EM are the most complete solution of bacteria in existence and are good in dealing with animal health (Jason, 2012). EM livestock products are all-natural, concentrated liquid probiotics containing a unique blend of naturally occurring beneficial bacteria. They are administered to livestock either as a feed additive or a concentrated dose for direct ingestion (Gaetane and Dylan, 2008).

The experiment conducted at Holetta Agricultural Research Center with the objective of ensiling crop residue and impact on milk yield by using EM reported that it improves the nutritive value, intake and digestibility of crop residues. Daily milk yield of lactating cows was also improved by feeding EM treated barley straw supplemented feed (Abera *et al.*, 2015).

There are reports those indicated that inclusion of live microorganisms in feed or water of chickens in adequate amounts significantly confers a health benefit on the host animals and improved survival rate. According to this report, 90% of the experimental chicks assigned to the treatment containing 12 ml of EM/liter of drinking water survived to an age of 4 weeks, the value of which is higher than all the others. The highest survival rate was recorded from male chicks placed on 12 ml of EM/liter of water to an age of 8 weeks. On the contrary significantly lower survival rate was recorded from the group paced on the control treatment of the same study population **(**Simeamelak, 2012**).** Hanekon *et al.* (2001) and Safalaoh and Smith (2001) reported that EM was successfully used for increasing survival rate in integrated animal units and poultry farms in South Africa. Improvement in health status of the birds seems to be attributed to the colonization of chicken intestinal tract by lactic acid bacteria which controls the population of pathogenic microorganisms such as *Salmonella, Enterococci* and *E. coli spp* (Edens *et al.* 1997).

2.5. Prevention and Control of *Salmonella* in Pre-harvest Production Chain

The various interconnected vehicles that may potentially transmit *Salmonella* on cattle operations discussed throughout this section make control of *Salmonella* extremely complex. Each vehicle has been documented as independently impacting the transmission of *Salmonella* to cattle as *Salmonella* is not only animal health concern but also food safety issue (Carlson *et al.,* 2011; Ge *et al.,* 2013; LeJeune *et al.,* 2001; Olafson *et al.,* 2014; Stephens *et al.,* 2007; Toth *et al.,* 2011; Toth *et al.,* 2013). A better understanding of the ecology of these microorganisms in natural setting will assist in developing interventions, which could aid in reducing the incidence and burden of *Salmonella*.

2.5.1. Direct- Fed Microbial Supplement

Direct-fed microbials (DFM) such as *Lactobacillus acidophilus* NP51 have been effective in mitigating the shedding of *Salmonella* in feedlot cattle when administered to cattle throughout the feeding period and prior to harvest at high doses (Stephens *et al.,* 2007). Pre-harvest interventions, such as DFM, can be implemented in conjunction with other sanitation procedures to create a multi-hurdle approach designed to control food borne pathogens throughout the beef production system (Callaway *et al.,* 2002). Unlike vaccination regimens, the inclusion of DFMs is relatively easy to incorporate into CAPFs by simply including them into the TMR. The use of DFMs has proven advantageous for multiple reasons: 1) DFMs have shown to effectively mitigate the shedding of *Salmonella* in feedlot cattle (Stephens *et al.,* 2007) and 2) producers often observe increased performance characteristics (e.g., weight gain and feed-to-gain ratio) in animals fed DFMs (Stephens *et al.,* 2007). The inclusion of DFMs are more widely adopted than vaccination regimens (Smith *et al.,* 2014; USDA-APHIS, 2009; USDA-APHIS, 2010; USDA-APHIS, 2013; Ison (2013) estimated 45.7% of feedlot-finished cattle harvested in 2012 were administered *L. acidophilus* NP51 at some point prior to harvest.

2.5.2. Vaccination

Vaccination aims to stimulate the development of naturally acquired immunity by inoculation of nonpathogenic, but still immunogenic, components of the pathogen in question (Meeusen *et al.,* 2007). Vaccines that induce protective immunity against colonization of pathogens may offer distinct advantages because of likely acceptance by cattle producers and ready incorporation into existing vaccination protocols (Loneragan and Brashears, 2005). Vaccination represents a sustainable, although minimally adopted, approach for promoting animal health, animal welfare, and food safety through mitigating pathogen exposure at the onset of commercial food production (Heithoff *et al.,* 2015; Mahan *et al.,* 2012). It has been reported that less than 1% of beef cattle operations utilize any type of commercially available *Salmonella* vaccine on their cattle (Mahan *et al.,* 2012), and less than 6% of animals fed in feedlots receive a *Salmonella* vaccine (USDA-APHIS, 2013). The latest statistic on the percentage of dairy farms that vaccinate against *Salmonella* reported by the USDA National Animal Health Monitoring System was 10% in 2007 (USDA-APHIS, 2009).

2.5.3. Animals wash

Prior to entering the abattoir, the hides of cattle are often contaminated with excrement, dust, and/or mud that frequently contain pathogenic bacteria (Bacon *et al.,* 2000; Brichta-Harhay *et al.,* 2011; Kalchayan *et al.,* 2009). This could be due to wind or muddy conditions at the time of shipping, the close confinement during transportation, the length of transport, and/or the facilities used for lairage (Dewell *et al.,* 2008; McEachran *et al.,* 2015; Reicks *et al.,* 2007). Carcass pathogen intervention systems have been widely studied; however, minimal research efforts have been directed toward the effects of intervention systems applied to animals prior to entry into the abattoir (Mies *et al.,* 2004).

2.6. *Salmonella* Prevention and Control in Postharvest Chain

2.6.1. Physical Intervention

Physical decontamination refers to removal of visible contamination on the carcass. This is accomplished using several methods including knife trimming, the use of ambient temperature water for rinsing the carcass, and steam-vacuuming. Knife trimming has been shown to be an effective method to remove visible contamination such as hair, fecal material, or ingesta. Prasai *et al*. (1995), excised samples of the surface of beef carcass sides in a commercial slaughter plant. These results indicate that trimming is an effective and acceptable corrective action for visible contamination, it is not sufficient in itself to remove all contamination, as microbial contamination is not visible (Prasai *et al.,* 1995).

2.6.2. Acid antimicrobials

Many acid antimicrobials are used in commercial beef plants as a means to reduce contamination. Organic acids are the more commonly used and studied agents. These include acetic, citric, and lactic acids. There are many factors that influence the effectiveness of these acids including concentration, pH and pKa (Baird-Parker, 1980). It is thought that these acids interfere with the transmembrane proton gradient of microbial cells and with structures of the cell surface, which disrupt nutrient transport and microbial growth (Brown and Booth, 1991; Corlett and Brown. 1980). Lactic acid is one of the most widely used organic acids in the meat industry due to a combination of effectiveness and cost (Wheeler *et al.,* 2014).

2.6.3. Oxidizer antimicrobials

Another category of post-harvest interventions is oxidizer antimicrobials. These can include peroxyacetic acid, electrolyzed oxidized (EO) water, or acidified sodium chlorite (ASC). Peroxyacetic acid is approved by the FSIS for use in commercial beef plants at a maximum of 1800ppm (FDA, 2015), although it is generally used at 200ppm. King *et al.* (2005), reported that use of peroxyacetic acid prior to chilling reduced *Salmonella* by 0.7 log10 CFU/cm2 on the carcass surface.

2.6.4. Thermal Interventions

Heat treatment is used as an intervention in many food processing environments including beef production. Hot water wash cabinets are common in beef processing plants as pre-evisceration and final carcass interventions (Wheeler *et al.*, 2014). Many studies have been conducted that investigated the use of water at temperatures ranging from 74oC up to 95oC. Spraying with hot water raises the temperature of the carcass surface. The FSIS acknowledges that water greater than 74oC will produce a sanitizing effect (Huffman, 2002). Arthur *et al.* (2008) reported that the use of a hot water (i.e. 74^{o}C) wash for 20 seconds reduced *Salmonella* contamination on the carcass by 1.04 to 2.10 log CFU/cm2.

2.6.5. Non- thermal Interventions

Non-thermal technologies like Ultraviolet (UV) light irradiations are either in use or are being investigated as alternative interventions. The UV light works by causing damage to DNA of microbes leading to cell death. The use of UV-C (wavelength of 220-300 nm with 90% of emission at 253.7 nm) has been approved by FDA for use on food products to control microorganisms (Chun *et al.*, 2010; FDA, 2007). Using UV-C is not expensive, and does not require the use of chemicals or heat. The effectiveness of UV-C light treatment against *Salmonella* has been reported on poultry

3. MATERIALS AND METHODOLOGY

3.1. Description of the Study area

The study was carried out in Oda Bultum district of Western Harerghea Zone, Oromia Regional State; Eastern Ethiopia. The specific site was Charcher Oda Bultum Farmers Cooperative Union (COBFU) farm, which is found in Gode-Hora Kebele. Oda Bultum district is located at approximately 375 km Far from Addis Ababa and 50km from zonal capital, Chiro. Geographically this area has an altitude of 1400 - 3100 m.a.s.l and the specific location of the site is provided bellow (*Fig: 1)*.The area has a mean temperature ranging from 22^0C - 28^0C. It receives an average annual rainfall of 900mm – 1200mm with bimodal distribution of the seasonal pattern peaking in mid-April and mid-August of the year; however there is a variation from year to year (OWAO, 2016). Currently, the farm is being operated with 200 local breed in fattening, 50 Cross breed and 140 Borena breed in dairy farms. The farm is equipped with production facilities like feed chopper, feed mixer, milking machine, basic veterinary equipment for clinical diagnosis and modern housing for both fattening and dairy which is suitable lay out for the purpose of the study.

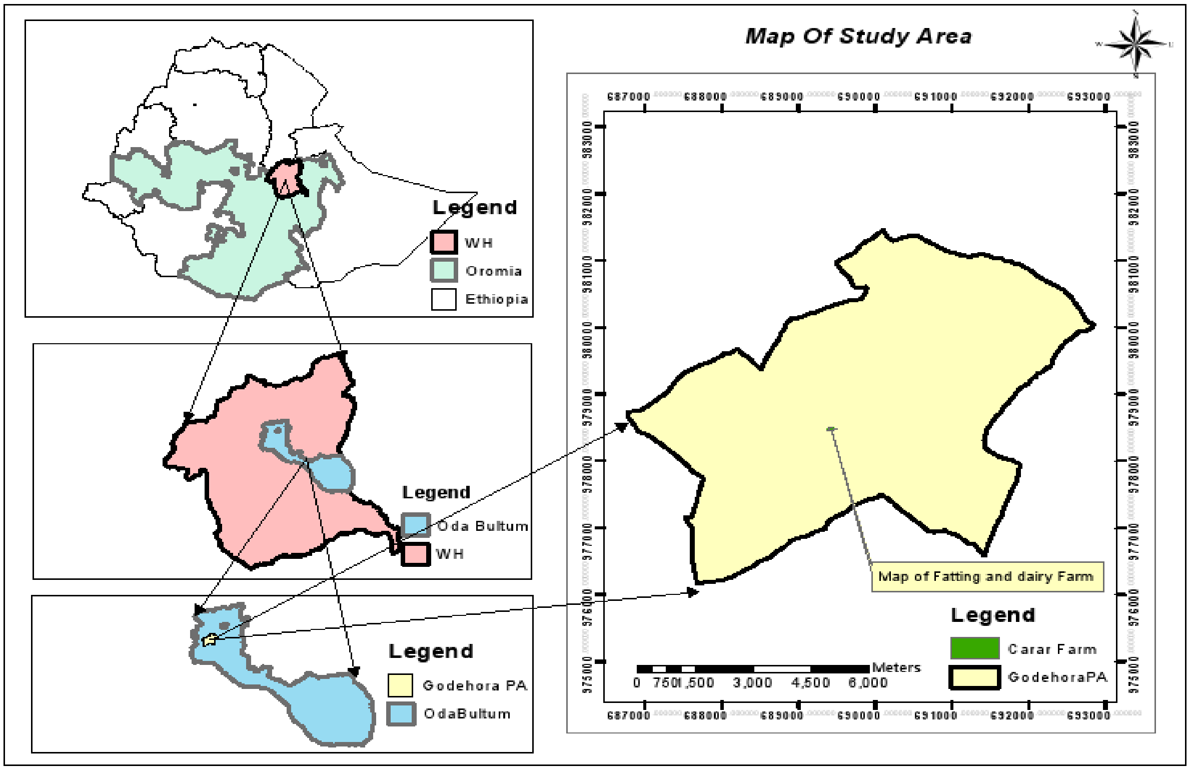

Figure 1. The Study Site Location in Ethiopia

3.2. Study Animals

In order to meet the specific objective, analogous to Vipham *et al.* (2015), the study were conducted in one commercial feedlot setting with two pen of 100 and 30 animals each. The farm is found in the study area stated above. All the bulls of study subjects were local zebu breeds of those mainly produced by the local small holders. They were bought from the local markets Baddessa town surrounded by highlands whereas Boke, Gabiba and Milkae were from Wabi-shebelle basin lowland areas of Harerghea where these three lowland areas are 30km, 60km and 120km distance from the study farm. The production system in low land is pastoral and in highland managed under zero grazing by the small holders (WHLFDO, 2016). The Chercher Oda-Bultum Farmers Union collect these animals for the purpose of finishing and supplying beef to abattoir or butchers of central Ethiopian markets like Addis Ababa, Mojo, Adama and large institutes including Haramaya and Oda –Bultum Universities.

For connivance of the following exposed and non-exposed animal at abattoir, all exposed animals and the randomized control groups, which were slaughtered at Haramaya University Abattoir, were examined for samples.

3.3. The Study Animals Management

Body condition scoring (Suiter 1994), and age determination of the study animals were done according to the standards developed by Canadian food inspection agency (AARD, 2013). Both control and treatment cohorts of feedlot cattle were received a starter diet and a finishing diet during the feeding period. The treatment diets were differed from the control cattle diet by the addition of EM·1® inoculants (EM Research Organization Japan, Inc. #3600-01-007771) and the control group was used molasses as placebo as the color of two liquids are similar as well as used as owner blinding.

The product was supplied by EM- Woljejii Agricultural Industry PLC, which is accredited distributer in domestic market. They were supplied in the form of feed mixed with EM2, molasses and warm water (chlorine free) in the ratio of 1:1:18 liter, according to manufacturer’s recommendation, with the target dose being 5×10^{10} cfu/day/head of *Lactobacillus*, 1×10^{9} cfu/day/head *yeasts*, 5×10^{5} cfu/day/head of *Phothosentethic bacteria* for 90,100 and 115 days based on batch of animals to be slaughtered. Treatment and control diets were administered for the duration of the feeding period and separate feeding trucks were used to administer the two different diets. Other than the treatment feed for treatment groups, the rest were the same in terms of natural challenge and local feed including hay, teff straw and “frushka” , coffee husk. Close supervision and monitoring were in place by using tools like checklists.

3.4. The Study Design

A Randomized Controlled Field Trial (RCFT) were conducted in which the treatment (EM .2) were randomly allocated either to treatment or control cohort with 100 and 30 animals/ pen where the two were parallel-group-designed at the beginning of the study considering animal age determination (according to the standards developed by Canadian food inspection agency), body condition (Suiter 1994), body weight, sources and exposure time. Hence, 100 and 30 animals were selected, tagged and registered for treatment and control respectively in each cohort. For these purpose, farmers and employed workers were trained on how to prepare and feed EM-microbial inoculants.

Following the study animals at study abattoir in both cohorts of the study, one sample of SLN and one sample MLN per carcass were collected from both treatment and control groups of animals at study abattoir immediately after slaughter. Thus, 130 samples of LNs each from both groups of animal (in total, 260 samples) were collected for laboratory examination. The cattle were slaughtered in three groups and therefore housed at the feedlot for 90, 100, and 115 days of exposure, respectively. Thus, pair of samples (SLN and MLN samples) from each the sampled animal were collected aseptically and separately.

3.5. Sample Size Determination

The prospective Randomized Control Field Trial of parallel groups designed study based on exposure to feed supplemented by EM.2 and non- exposed group to EM.2.

Sample size was calculated by using the formula given by Arsham (2002)[*] which is:

$$N = 0.25/SE^2,$$

Where: N= sample size, SE (standard error) = 5%); Hence, the required sample sizes were 100 animals for treatment and 30 for control cohort.

Thus, a total of 130 animals used for commercial purpose were used as convenience sampling as it were accessible, manageable and convenient for group harvesting and sample collection within the project time frame. The limitation of resource and its irrationality to buy and slaughter those numbers of animals for this project was another rationale to use the same animals for both research and commercial purpose.

3.6. Sampling

Following specific identification given during the feeding, the samples of SLN and MLN were aseptically collected and registered with same identification code used while animals were alive at the farm. The samples were transported to Veterinary Microbiology Laboratory, College of Veterinary Medicine, Haramaya University for immediate process on the date of sampling. Sample collection and processing were done aseptically (flaming the sampled LNs before processing) but blinded using the coding system that has been given at the beginnings of study. Thus, codes were lifted in to Excel sheet after data collection in order to conduct statistical analysis.

3.7. Laboratory Sample Analysis

3.7.1. *Salmonella* Isolation and Characterization

Sampled LNs were processed as previously described (Gragg *et al.*, 2013; Vipham, 2015). Surrounding fat and fascia were trimmed from LN samples, which were weighed, surface sterilized by surface flaming, placed into individual filtered sample bags, and pulverized using a stomacher (model 400 stomacher, Seward, Worthington, UK) at 230 rpm for 2 minutes. The isolation and identification of *Salmonella* were undertaken following conventional cultural methods. Briefly, each processed sample was pre-enriched in BPW (BM020, Sisco Research Laboratories; India), (1: 9) and incubated for 16–20 h at 37^{o}C. From the pre-enrichment broth, 100 micro liters were transferred into 9.9 ml of Rappaport Vassilliadis (RV) (Oxoid) broth and incubated at 42^{o}C for 24 h. A loop full of the inoculums from RV was streaked side by side onto Xylose Lysine Deoxycholate agar (XLDP) (M031 – 500G, HiMedia Laboratories Pvt. Ltd), and brilliant green phenol red lactose sucrose (BPLS) (Merck) agar plates and incubated at 37^{o}C for 24 h. The Presumptive *Salmonella* colonies were colonies were purified on fresh nutrient agar (HiMedia, India) and further characterized using conventional biochemical tests. Isolated *Salmonella* colonies were inoculated onto triple sugar iron agar (TSI) (M021-500G, HiMedia Lab. Pvt. Ltd, India) lysine iron agar (LIA) (CM081, Oxoid LTD, England) Simmon's citrate (M099, HiMedia Lab. Pvt. Ltd, India) and Moreover, two or more colonies from pure isolates were inoculated on urea broth (SRL, India) and incubated at 37^{o}C for 24 h for conformation according to (Grimont and Weill, 2007).

3.7.2. *Salmonella* Enumeration

Quantitative culture methods were conducted according to Gragg *et al.,* (2013), where one ml of the TSB/SLN homogenate were removed prior to initial incubation, plated in duplicate onto counting plate /*Enterobacteriaceae* (EB) count plates (EB; Petrifilm™, 3M, St Paul, MN, USA) and incubated for 22-26 hours at 37°C. Petrifilm plates were then held at 4^{0} C until presumptive culture results were obtained. Colonies were counted with colony counter according to manufacturer's instructions and recorded considering minimum 30 CFU and maximum 100 CFU per plate was counted. Each of the separate colony of bacterial growth on EB count plates (petrifilm™) were transferred to XLD (M031 – 500G, HiMedia Laboratories Pvt. Ltd), agar and incubated for 16 hours at 37°C. Morphologically typical colonies on XLD plates were counted and comparisons were made with EB count plate (petrifilm™) counts. Concentrations of *Salmonella* were reported on a cfu / 25g of lymph node basis.

3.8. Data Analysis

Raw data were interred to Microsoft Excel 2007© and analyzed using STATA 12.1. For qualitative data, r/n (events/trials) binomial response variables was created for each control and treatment groups, where r is the number of positives and n is the number of lymph nodes to be assayed. Cohort study risk ratio was used grossly at pen level relative risk estimation. Multi level-mixed effects Model, extension to Mixed- effects linear regression was constructed in which risk factors were considered a random variable and used to screen the potential confounding factors. Model estimation was achieved using maximum likelihood method and Wald Chi^{2}. To account for potential within and among pen dependency residuals (i.e., clustered outcomes) a random effect regression model was used. The mean prevalence for the treatment by time interaction was analyzed and data were used for estimation of percent efficacy. Relative risks (RR), relative risk reductions (RRR) and absolute risk (AR) at 95% confidence intervals (C.I. 95%) were calculated where ($P < 0.05$) was considered as significant *Salmonella* prevalence and load.

Concentration data were transformed to log10 and analyzed. The result was expressed using mean and standard deviations in common logarithmic function based on the types of LN (SNL and MLN), time of exposure (harvesting days). Linear regression and single t-test were used to determine mean logarithm of count among pens, types of samples (LNs) and day of harvest at 95% CI, where ($P < 0.05$) was again considered as significant association.

4. RESULT AND DISCUSSIONS

4.1. *Salmonella* in the Study Animals

The total number of lymph nodes (N = 260), where, n = 130 and 130 of them were MLN and SLN respectively; 200 were collected from 100 cattle administered EM2 (Treatment) and 60 were collected from 30 cattle in the control group. A total of 33/100 (33%) and 21/30 (70%) LNs collected from cattle in the treatment and control groups were found *Salmonella* positive respectively. A greater percent of positives (70%) were observed LNs collected from cattle in the control group (Table 1).

A significant reduction in *Salmonella* prevalence in LNs (MLN and SLN) was observed from cattle administered EM2 with a relative risk reduction (RR: 0.53; 95% CI = 0.3, 0.68, p = 0.00). The risk of *Salmonella* harborage in LNs of EM supplemented group was 53% less than the counterpart and 37% of the risk could be reduced by EM supplementation (Table 2). On a percentage basis the amount of positive animals in the treatment group was 33%. A higher percentage of positives 70% were observed in cattle from control group. This result agree with the slight difference in treatment group from similar study by Viamp *et al.* (2015) that report prevalence of 57% and 76.3% in treatment and control respectively.

Table 1. The effect of Feeding EM in Reduction of *Salmonella* in specific Lymph Node of Studied Animals

Measures Effect Variables	**No of study animals**	**Proportion of *Salmonella* 95% CI**	**χ2**	**P- Value**
Non-Treated Group(Control)	30	21/30 (0.70), [0.51 - 0.85]	13.01	0.000
EM Feed Group (Treatment)	100	33/100 (0.33), [0.24 – 0.43]		
Risk Difference (Absolute Risk)	*	0.37 (0.17 - 0.57)		
Relative Risk	*	2.12 (1.41 - 3.20)		
Relative Risk Reduction	*	0.53 (0.29 - 0.69)		

In this study relative reduction in the prevalence of *Salmonella* within MLN and SLNs was observed in the study cattle presented for harvest after treated by EM. The data reported herein indicate that administering Effective Microorganisms (EM) to cattle during the feeding period has an effect in reduction of *Salmonella* detected in MLN and SLNs of beef cattle. These preliminary data are impactful for the beef industry as well as public health, since lymph nodes, including SLNs within beef are commonly incorporated into beef trim destined for ground beef production.

As shown in (Table 2), variable proportion of *Salmonella* was observed in MLN and SLN of both control and EM-feed animals except for those EM-feed for 115 days those with good body condition. As age of animals increases the prevalence of *Salmonella* become increasing in both MLN and SLN of control groups but reduction in SLN of EM-feed animals were observed. Concomitant raise in body condition and body weight of animals leading to reduction in prevalence of *Salmonella* were observed regardless of treatment type. Significant reductions in Salmonella prevalence with increase in treatment time for EM-feed group were observed.

Table 2. Prevalence of *Salmonella* in Specific Lymph Node across Studied Risk Factors in Studied Animals

	Proportions of *Salmonella* positive in (MLN)		**Proportions of *Salmonella* positive in (SLN)**	
Risk factors	**Control**	**Treatment**	**Control**	**Treatment**
Age (Years)				
2 -3.5	6/11 (0.55)	6/37 (0.16)	3/11 (0.27)	8/37 (0.22)
3.5 - 4.5	6/12 (0.50)	9/55 (0.16)	8/12 (0.67)	12/55 (0.23)
> 4.5	7/7 (1.00)	2/8 (0.25)	5/17(0.71)	1/8 (0.13)
Body Condition				
Poor	11/14 (0.78)	6/35 (0.17)	9/14 (0.64)	11/35 (0.31)
Medium	6/10 (0.60)	10/55 (0.18)	7/10 (0.70)	8/55 (0.15)
Good	2/6 (0.33)	1/10 (0.10)	0/6 (0.00)	2/10 (0.20)
Body weight				
177 - 200	7/13 (0.54)	8/40 (0.20)	6/13 (0.46)	10/40 (0.25)
200 - 214	9/11 (0.82)	2/15 (0.13)	8/11 (0.72)	4/15 (0.27)
214 - 225	3/6 (0.50)	7/45 (0.16)	2/6 (0.33)	7/45 (0.16)
Animals Source				
Low Land	10/17 (0.59)	12/61 (0.19)	10/17 (0.59)	15/61 (0.25)
Highland	9/13 (0.69)	5/39 (0.13)	6/13 (0.46)	6/39 (0.15)
Treatment time				
90 days	6/9 (0.67)	11/33 (0.33)	5/9 (0.56)	12/33 (0.36)
100 days	6/9 (0.67)	6/31 (0.19)	6/9 (0.67)	8/31 (0.26)
115 days	7/12 (0.58)	0/36 (0.00)	5/12 (0.42)	1/36 (0.03)
Total poroportion	19/30 (0.63)	17/100 (0.17)	16/30 (0.53)	21/100 (0.21)
95 % CI	[0.43 - 0.80]	[0.10 - 0.26]	[0.34 - 0.72]	[0.13 - 0.30]

4.1.1. *Salmonella* Risk Reduction in Misentric Lymph Node (MLN)

Variations in positive MLNs by other risk factors like age group, body condition, body weight, source of animals and duration of time in treatment were observed, with 7/7 (1.0) in age group above 4.5 and 2/8 (0.25) in the treatment group of the same age. Regards to body condition in poor 11/14 (0.78) and 6/35 (0.17) MLN were positive in control and treatment respectively (Table 1). However, statistically significant reductions were associated with EM supplement, duration of treatment and age groups (Table 2).

Regardless of few number current sample the 70% observed in the control group revealed the natural S*almonella* history of the farm and higher compared to 23.5% from animal feces by Hiko *et al.* 2016 and 19% found in rumen contents reported by Sibhat *et al*. (2009). The difference among these reports might be attributable to the sample type in the current study was lymph nodes where bacteria concentrated due to the action of immune system and the others were at carcass level. In addition, *Salmonella* are versatile enteric pathogens noted for their ability to invade and survive within host lymphoid tissues (Durand *et al.*, 1990). In the current study, we also observed that *Salmonella* could be recovered from MLN of positive cases. Those from the control group, indicate the infection status of the animals.

A significant reduction in *Salmonella* prevalence in MLN was observed from cattle administered EM with a relative risk reduction of (RR: 0.73; 95% CI = 0.55, 0.84, $\chi 2$ = 24.74 and p = 0.00), (Table 3). Whereas, significant differences were also observed across time of treatment (Days of harvesting) but not the first day (90 days) treatment (P = 0.070). The relative risk reduction on second day (100 days) of treatment (RR: 0.71, 95 % CI **=** 0.32, 0.88, $\chi 2$ = 7.43 and P = 0.006) and relative risk reduction (RR = 1.00, where, ($\chi 2$ = 24.59 and P = 0.000**)** on the third (115 days), (Table 5). There were also significant absolute risk reduction in age groups (P = 0.000) in cattle age of (2 _ 3.5), (3.5 _ 4.5) and (> 4.5) yrs respectively (Table 6). We haven’t come across with the report specific to the effect of EM on *Salmonella* in MLN this might be related to its low food safety importance.

Therefore, this directs us to the hypothesis on the potential modes of actions by which *Lactobacilli* exert their protective or therapeutic effect. The *lactobacilli* achieve this effect through production of antimicrobial compounds (Dodd and Gasson, 1994), reduction of gut pH by stimulating the lactic acid producing microflora (Langhendries, 1995), competition for binding of receptor sites that pathogens occupy (Kailasapathy and Chin, 2000), stimulation of immunomodulatory cells (Rolfe, 2000). Demeria *et al.* (2009) support this observation by indicating that many strains of *Lactobacillus* are capable of eliciting different immune responses; from enhanced epithelial resistance to increased antibody production and competition with pathogens for available nutrients (Rolfe, 2000). The EM in current study might have done one or more of actions listed above. Generally, it is important to note the complexity of the bovine lymphatic system in order to fully understand the limitations of our data and the inferences that can be made from it. Further investigations into the ecology of *Salmonella* within the bovine lymphatic system should be a goal for future research and will provide a more in-depth understanding of this issue.

Table 3. The Effect of Feeding EM on *Salmonella* in Mecentric Lymph Node (MLN)

Measures Effect Variables	**No of study Animals**	**Proportion of *Salmonella* Positive (95% CI)**	**$\chi 2$**	**P- Value**
Non-Treated Group(Control)	30	19/30 (0.63), [0.44 - 0.80]	24.74	**0.000**
EM Feed Group (Treatment)	100	17/100 (0.17), [0.10 - 0.26]		
Risk Difference (Absolute Risk)	*	0.46 (0.28 - 0.65)		
Relative Risk	*	3.72 (2.21 - 6.27)		
Relative Risk Reduction	*	0.73 (0.55 - 0.84)		

Table 4. The *Salmonella* Risk Difference across Risk Factors in Misentric Lymph Node

Variable	**Risk Difference**	**P > /Z/**	**95% CI**	**Wald (χ2)**	**P _ Value**
Pen	0.43	0.00	0.26 - 0.59	**56.71**	**0.000**
Age	0.29	0.004	0.009 - 0.049		
Body Condition	0.009	0.364	0.011 - 0.031		
Source	0.003	0.963	0.141 - 0.134		
Weight	0.004	0.571	0.01 - 0.02		
Time	0.023	0.001	0.009 - 0.035		

Table 5. Effect of Feeding EM on *Salmonella* Reduction Across Time in Mecentric Lymph Node of Studied Animals

	Proportion of *Salmonella* Positive Animals on Days of Harvesting		
Treatment category	**90 days**	**100 days**	**115 Days**
Prevalence of *Salmonella* (Control)	6/9 (0.67), [0.30 - 0.93]	6/9 (0.67), [0.30 - 0.93]	7/12 (0.58), [0.28 - 0.85]
Prevalence of *Salmonella* (Treatment)	11/33 (0.33), [0.18 - 0.52]	6/31 (0.19), [0.07 - 0.37]	0/36 (0.00) -
Risk Difference (Absolute Risk)	0.33 [- 0.014 - 0.68]	0.47 [0.14 - 0.81]	0.58 [0.30 - 0.86]
Relative Risk	2.00 [1.03 - 3.90]	2.47 [1.47 - 8.09]	*
Relative Risk Reduction	0.50 [0.029 - 0.74]	0.71 [0.32 - 0.88]	1.00
χ2; p - Value	3.26, 0.070	7.43; 0.006	24.59; 0.000

Table 6. The Salmonella Risk Difference Across Age in Misentric Lymph Node

Age	Risk Difference	P > /Z/	95% CI	Chi2	p- Value
2 - 3.5	0.38	0.005	0.11 - 0.65	**7.71**	**0.000**
3.5 - 4.5	0.34	0.008	0.08 -0.58		
> 4.5	0.75	0.000	0.43 - 1.1		

4.1.2. *Salmonella* Risk Reduction in Sub iliac Lymph Node (SLN)

The trend of *Salmonella* prevalence in SLN also varies across the blocks of risk factors with 5/17 (0.71) and 1/8 (0.13) for cattle above 4 years of control and treatment groups respectively in the same age. Variations in positive SLNs by risk factors were observed, with treatment group having reduction to as few as 1 positive (0.03%) 115th days of treatment but as many as 12 positive (0.36%) in the same category of 90th day. The variation was observed amongst risk factors in the control group with 0 positive (0.00%) as many as 7 positive (70%) in the same group. The total prevalence of *Salmonella* in SLN was 16/30 (0.53) in control and 21/100 (0.21) in treated group (Table 2).

The previous researches have shown that cattle peripheral LNs including SLN can serve as a vehicle for *Salmonella* contamination; if fat trim containing these nodes are incorporated into ground beef directly have food safety implication (Adhikari *et al.*, 2004; CDC, 2012). Contaminated LNs may explain the difference in *Salmonella* prevalence between post-intervention carcasses or trim, and ground beef (Arthur *et al.* 2008). The current studies 53% prevalence of *Salmonella* in SLN of control group might confirm the idea above theorized by (Arthur *et al.* 2008). In that it was higher than 32.4% from row beef at butchery reported by Hiko *et al.* 2016 as well as 40% and 42% prevalence reported from minced meat (locally known as «kitfo») while the samples were collected from different hotels, bars and restaurants in Addis Ababa (Molla *et al.*, 2000; Tegegne and Ashenafi, 1998). This finding was comparable with the

60% rate found among samples from a South African slaughterhouse (Nel *et al.*, 2004), and lower than the 87.4% rate reported by Stevens *et al*. 2006 from retail beef in Senegal.

However, the statistical significances were associated with treatment (EM) and time of treatment (Table 8 and 9). A significant reduction in *Salmonella* prevalence in SLNs were observed from cattle administered EM with a relative risk reduction (RR: 0.61; 95% CI = 0.33, 0.77, χ2 = 11.85 and p = 0.00). The EM supplemented group had 61% less likely to harbor *Salmonella* in their SLNs compared to non-supplemented ones as well as 32% risk reduction was attributable to EM (Table 7). On the other hand as shown in (Table 9), the effect varied across slaughter days (Time of treatment) with no significant reduction (χ2 = 1.08, P = 0.298) on first day. However, significant differences were observed on the second and third days with (RR: 0.61, 95% CI = 0.18, 0.82, χ2 = 5.12; P = 0.023) and (RR: 0.93, 95% CI = 0.48, 0.99; χ2 = 12.44, P = 0.000) respectively (Table 9). To our knowledge there is no data available in Ethiopia making it difficult to create meaningful comparison of interaction observed in this study in domestic. The limited number of the animals blocked to different risk factors based on biological difference in the beef farm is a limiting factor in our ability to make inferences to Ethiopia even if we consider grouping of cattle in the study farm according to the source population, and it is important when interpreting these data to consider this limitation. Considering the absolute risk reduction between the treatment and control study animals allows for a better frame of reference for interpretation.

However, the current study reduction trends agree with the 50%, 31% and 10% across three slaughter days by Viamph *et al.*, (2015) from USA. The slight difference lay on the difference in duration of the treatment which is 90 days based on the fattening package in Ethiopia and above 129 days in USA that might be due to agro ecological and beef breed difference.

However, in current study the EM supplemented group was 39% less likely to be infected by *Salmonella* (RR: 0.61, 95% CI = 0.33, 0.77), the finding agrees in principle with 82% reported by Viamph *et al.*, (2015) in USA and lower in magnitude of effect. The difference shown might be attributed to the ground and setting of study animals in which the current study subjects were in natural challenge and the later in research farm as well as high difference in sample size among the two studies.

Table 7. The Effect of Feeding EM on *Salmonella* in Subiliac Lymph Node

Measure Effect Variables	**No of study Animals**	**Prevalence of *Salmonella* (%), 95% CI**	**χ2**	**P- Value**
Non-Treated Group(Control)	30	16/30 (0.53), [0.34 - 0.72]	**11.85**	**0.000**
EM Feed Group (Treatment)	100	21/100 (0.21), [0.13 - 0.30]		
Risk Difference (Absolute Risk)	*	0.32 (0.14 - 0.51)		
Relative Risk	*	2.54 (1.49 - 4.33)		
Relative Risk Reduction	*	0.61 (0.33 - 0.77)		

Table 8. The *Salmonella* Risk Difference across Risk Factors in Subiliac Lymph Node

Variable	**Risk Difference**	**P > /Z/**	**95% CI**	**Chi2**	**P - Value**
Pen	0.308	0.001	0.133 - 0.48	**33.99**	**0.000**
Age	0.013	0.232	(-)0.034 - 0.008		
Body Condition	0.012	0.274	(-)0.009 - 0.035		
Source	0.049	0.511	(-)0.098 - 0.197		
Weight	0.007	0.351	(-)0.008 - 0.022		
Time	0.024	0.001	0.01 - 0.038		

Table 9. The Effect of Feeding EM on *Salmonella* Reduction across Time in Subiliac Lymph Node

	Proportion of *Salmonella* Positive animals and Days of Harvesting		
Treatment category	**90 days (95% CI)**	**100 days (95% CI)**	**115 Days (95% CI)**
Prevalence of *Salmonella* (Control)	5/9 (0.56), [0.21 - 0.86]	6/9 (0.67), [0.30 - 0.93]	5/12 (0.42), [0.15 - 0.72]
Prevalence of *Salmonella* (Treatment)	12/33 (0.36), [0.20 - 0.55]	8/31 (0.26), [0.12- 0.45]	1/36 (0.03), [0.001 - 0.15]
Risk Difference (Absolute Risk)	0.19 (-0.17 - 0.56)	0.41(0.06 - 0.75)	0.39 (0.10 - 0.67)
Relative Risk	1.53 (0.73 - 3.19)	2.58 (1.21 - 5.49)	15 (1.94 - 115.9)
Relative Risk Reduction	0.35 (-0.37 - 0.68)	0.61 (0.18 - 0.82)	0.93 (0.48 - 0.99)
χ2; p- Value	1.08, 0.298	5.12; 0.023	12.44; 0.000

4.2. Load of *Salmonella* in Lymph Nodes of the Study animals

The study demonstrated a shift in load of *Salmonella* in LNs (MLN and SLNs) due to the influence of supplementation EM. Higher load of *Salmonella* were observed in both MLN and SLNs from cattle in the control group than in those had been supplemented with EM. Variation in *Salmonella* mean load among all risk factors were absorbed with the statistical significance associated with treatment (EM), duration of treatment and age groups in MLN (p = 0.000) (Table 10). Whereas EM treatment and its duration (p = 0.000), as well as body condition score (p = 0.025) in SLN (Table 12).

4.2.1. The Load of *Salmonella* in Micentric Lymph Node (MLN)

Significant interaction was observed across three categories of days between load and slaughter day on a cfu/25 g of lymph node basis with the mean difference of (0.895 ± 0.26; 95% CI = 0.389, 1.40; Chi2 = 12.02; p=0.000), (1.39 ± 0.28; 95% CI = 0.83, 1.95, Chi2 = 23.73, p = 0.000), (3.30 ± 0.56; 95% CI = 2.20, 4.40, Chi2 = 34.34, p = 0.000) on the 90 th day, 100th day and 115th days respectively (Table 11). The interaction of *Lactobacillus acidophilus* with pathogenic bacteria specific to MLN was not well documented. This direct us to former hypotheses on potential modes of action for *Lactobacillus* including production of antimicrobial compounds (Dodd and Gasson, 1994, reduction of gut pH by stimulating the lactic acid producing microflora (Langhendries, 1995), competition for binding of receptor sites that pathogens occupy (Kailasapathy and Chin, 2000), stimulation of immunomodulatory cells (Rolfe, 2000). Demeria *et al.* (2009) support this observation by indicating that many strains of *Lactobacillus* are capable of eliciting different immune responses; from enhanced epithelial resistance to increased antibody production and competition with pathogens for available nutrients (Rolfe, 2000). Edens *et al.* (1997) reported that the supplementation of EM in poultry feed improved the health status of the birds and that might be attributed to the colonization of chicken intestinal tract by Lactic acid bacteria which controls the population of pathogenic microorganisms such as *Salmonella*, Enterococci and *E. coli spp*. The study on rat model in Nigeria, reported by Oyetayo *et al.*, (2003), by histopathological analysis confirmed the protective effect of the *lactobacillus.* The protection of the GIT was observed in rats treated with *Lactobacillus,* where the villus patterns of the small intestine of the rats were well preserved and count of enterobacteria were substantially reduced in the faces of rat model.

Table 10. The *Salmonella* Mean Log 10 cfu / 25 g of Lymph Node Difference across Risk Factors in Mecentric Lymph Node

Variable	**Mean Difference**	**P > /Z/**	**95% CI**	**Chi2**	**P _ Value**
Pen	1.34 ± 0.18	0.000	0.99 - 1.69	**111.80**	**0.000**
Age	0.11 ± 0.02	0.000	0.071 - 0.151		
Body Condition	0.013 ± 0.028	0.627	(-)0.067 - 0.041		
Source	0.19 ± 0.195	0.332	(-)0.19 - 0.57		
Weight	0.033 ± 0.021	0.117	(-)0.008 - 0.075		
Time	0.093 ± 0.02	0.000	0.053 - 0.133		

Table 11. The *Salmonella* Mean Log 10 cfu/ 25 g of Lymph Node Difference across Time and Age in Mecentric Lymph Node

Variables	**Mean Difference**	**P>/Z/**	**95% CI**	**Chi2**	**P _ Value**
Duration of Treatment					
90 days	0.895 ± 0.26	0.001	0.38 - 1.40	12.02	0.000
100 days	1.39 ± 0.28	0.000	0.83 - 1.95	23.73	0.000
115 days	3.30 ± 0.56	0.000	2.20 - 4.40	34.34	0.000
Age groups					
2 _ 3.5 yrs	1.54 ± 0.30	0.000	0.95 - 2.14	25.74	0.000
3.5 _ 4.5 yrs	1.21 ± 0.26	0.000	0.70 - 1.73	21.49	0.000
> 4.5 yrs	1.23 ± 0.35	0.000	0.54 - 1.92	12.15	0.001

4.2.2. The Load of *Salmonella* in Subiliac Lymph Node (SLN)

The *Salmonella* load reduction by 1.34log10 cfu/ 25g in SLN was attributable to the supplementation of EM to the diet of beef cattle (Table 10). The interactions were observed between load and slaughter day on a cfu/ 25g lymph node basis in the control and those had been supplemented with EM during the study period on the 90, 100 and 115th days of slaughtering with the mean difference (0.23 ± 0.26; 95% CI = 0.013, 1.04; Chi2 = 4.04 and P = 0.045), (0.89 ± 0.27, 95% CI = 0.37, 1.41; Chi2 = 11.14; P = 0.001) and (1.23 ± 0.31, 95% CI = 0.62, 1.85; Chi2 = 15.39; P = 0.000) log10 cfu/ 25g lymph node respectively (Table 13). The trend in reduction of *Salmonella in* log $_{10}$ in the current study is in agreement with Viamph *et al.* (2015) reported 2.78 log10 in USA. Beyond the reduction trend, for the magnitude difference between the studies it is also important to consider the differences between the studies as far as sample size cattle breed and management protocol of the farms involved in the study.

Table 12. The *Salmonella* Mean Log10 cfu/ 25g of Lymph Node Difference across Risk Factors in Subiliac Lymph Node

Variable	**Mean Difference**	**P > /Z/**	**95% CI**	**Chi2**	**P -Value**
Pen	0.80 ± 0.17	0.000	0.475 - 1.13	**54.43**	**0.000**
Age	0.040 ± 0.22	0.066	(-)0.003 - 0.084		
Body Condition	0.083 ± 0.037	0.025	0.011 - 0.156		
Source	0.196 ± 0.178	0.27	(-)0.152 - 0.544		
Weight	0.036 ± 0.019	0.069	(-)0.003 - 0.074		
Time	0.064 ± 0.018	0.000	0.03 - 0.098		

Table 13. The *Salmonella* Mean Log10 cfu/ 25g of Lymph Node Difference across Time and Body Condition in Subiliac Lymph Node

Variables	**Mean Difference**	**P>/Z/**	**95% CI**	**Chi2**	**P _ Value**
Duration of Treatment					
90 dys	0.895 ± 0.26	0.001	0.38 - 1.40	12.02	0.000
100 days	1.39 ± 0.28	0.000	0.83 - 1.95	23.73	0.000
115 days	3.30 ± 0.56	"	2.20 - 4.40	34.34	0.000
Body Condition Score					
Poor	1.54 ± 0.30	0.000	0.95 - 2.14	25.74	0.000
Medium	1.21 ± 0.26	"	0.70 - 1.73	21.49	0.000
Good	1.23 ± 0.35	"	0.54 - 1.92	12.15	0.001

5. SUMMARY CONCLUSSIONS AND RECOMMENDATIONS

The controlled field trial was conducted at Chercher Odabultum Farmers Union Farm with objectives to find out the effect of Effective Microorganisms (EM) supplementation in the diet of beef cattle on the reduction *Salmonella* in lymph nodes of live animals. The study was conducted on bulls of local zebu breeds those mainly produced by the local small holders and collected for finishing and beef supply to Haremaya University. The bulls were randomly allocated to control and treatment group where both cohorts were received the same local diet but EM was supplemented to the treatment group's diet. They were supplied in the form of feed mixed with EM, molasses and warm water (chlorine free) in the ratio of 1:1:18 liter, with the target dose being $\times 10^{10}$ cfu/day/head of *Lactobacillus,* 1×10^{9} cfu/day/head *yeasts,* 5×10^{5} cfu/day/head of *Phothosentethic bacteria* for the study period. Both treatment and control group were managed under the natural challenge and supplied 'firushka, teff straw hay and coffee husk'. The cattle were slaughtered at Haremaya University abattoir in three batches, on 90th, 100th and 115th days of the feeding period. Lymph node (MLN and SLN) sample were collected and immediately laboratory analysis for *Salmonella* isolation, characterization and enumeration were conducted.

Results of the trial revealed that reduction in *Salmonella* prevalence in LNs (MLN and SLN) was observed from cattle administered EM. The data reported herein indicate that administering Effective Microorganisms (EM) to cattle during the feeding period has an effect on the prevalence and burden of *Salmonella* detected in MLN and SLNs of beef cattle. However, statistically significant reductions were associated with EM supplementation, duration of treatment and age groups. The trial data showed that feeding EM for 100 days and above were responsive in reduction of *Salmonella* prevalence from lymph nodes (MLN and SLN) of live animals at pre-harvest. However, in case of SLN age didn't have interaction with the effect of EM. The study demonstrated a shift in load of *Salmonella* in LNs (MLN and SLNs) due to the influence of EM supplementation. Lower load of *Salmonella* were observed in both MLN and SLNs from EM treated cattle. The treated cattle were responsive on 90th days and above in the reduction of *Salmonella* burden in the lymph node of live animal. However, the current study is point in time with its own limitations, so it needs continuous investigation on the efficacy of this biotechnology across time due to the fact that, as the duration of exposure increased there might be an equilibrium occurring in the animal's microbiota, diminishing the effect of the initial response. In addition, it needs to consider the biological maturity of animals for fattening. Like the treatment age has strong association with the effect of EM in MLN where body condition has positive interaction with the effect of EM in the SLN.

In this study, a consistent and absolute reduction in the prevalence and load of *Salmonella* within LNs (MLN and SLN) of cattle feed with EM and presented for harvest destined for human consumption were observed. These preliminary data are impactful for the beef industry as well as public health, since lymph nodes, including SLNs, have high chance of incorporation into beef trim destined for ground beef production. The prevalence and load data in case of control and MLNs demonstrated that the burden of infection under natural history of *Salmonella* in beef farms of the study location.

Results from this study indicate that the application of EM as a pre-harvest intervention will aid in reducing *Salmonella* prevalence and load in bovine LNs (MLN and SLNs) in feedlot farm setting. Although the literature reveals a gap in the research available for the effects of EM on *Salmonella* in lymph node, our findings are consistent with the current understanding of the efficacy of this biotechnology in reducing pathogen carriage in ruminant systems. Further, these data support previously cited studies (Viamph *et al.* 2015), that have shown that feeding *Lactobacillus animalies* reduces pathogen shedding by animals in feedlot settings, thus reducing pathogen loads entering harvest facilities as well as ground beef processing facilities. Successful pre-harvest interventions such as Effective Microorganisms (EM) can possibly aid in outbreak prevention, reduce recall costs not only for the industry but the government as well, and strengthen protection of public health.

Although the current study suggests that this biotechnology is promising as a pre-harvest in intervention, all what has been described were in relative term. It means there was not complete illumination of *Salmonella* even in a treated group of study animals. Therefore;

- Consistent investigations in different agro ecology, season, breeds and production system of the country should be conducted.

- Investigation of the routes of entry by using animal models and diagnostic methods like Pulse Field Gel Electrophoresis (PFGE) should be done
- The interaction of EM and the pathogen *Salmonella* and mechanisms of action within complete bovine immune system should be fully investigated
- The mode of action of EM on *Salmonella* and other related pathogens in connection with ruminants' immune system should be studied.
- Routine hygienic animals production could also used as synergy with EM
- Interactions of EM and its efficacy across time considering the national fattening package as well as market season should be investigated

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8. ANNEXES

Annex 1. Body Condition Categorization

Good	Medium	Poor
Back bone can be felt but smooth and round, short ribs are smooth and well covered, eye muscle are rounded and full.	Back bone prominent but smooth, short ribs are smooth and fell, eye muscle have reasonable depth with surface feeding to feel flat.	Back bone prominent and sharp, short ribs ends sharp and easily to pres between over and around, eye muscle are thin, the surface feeding to feel hollow.

Source: (Suiter, 1994).

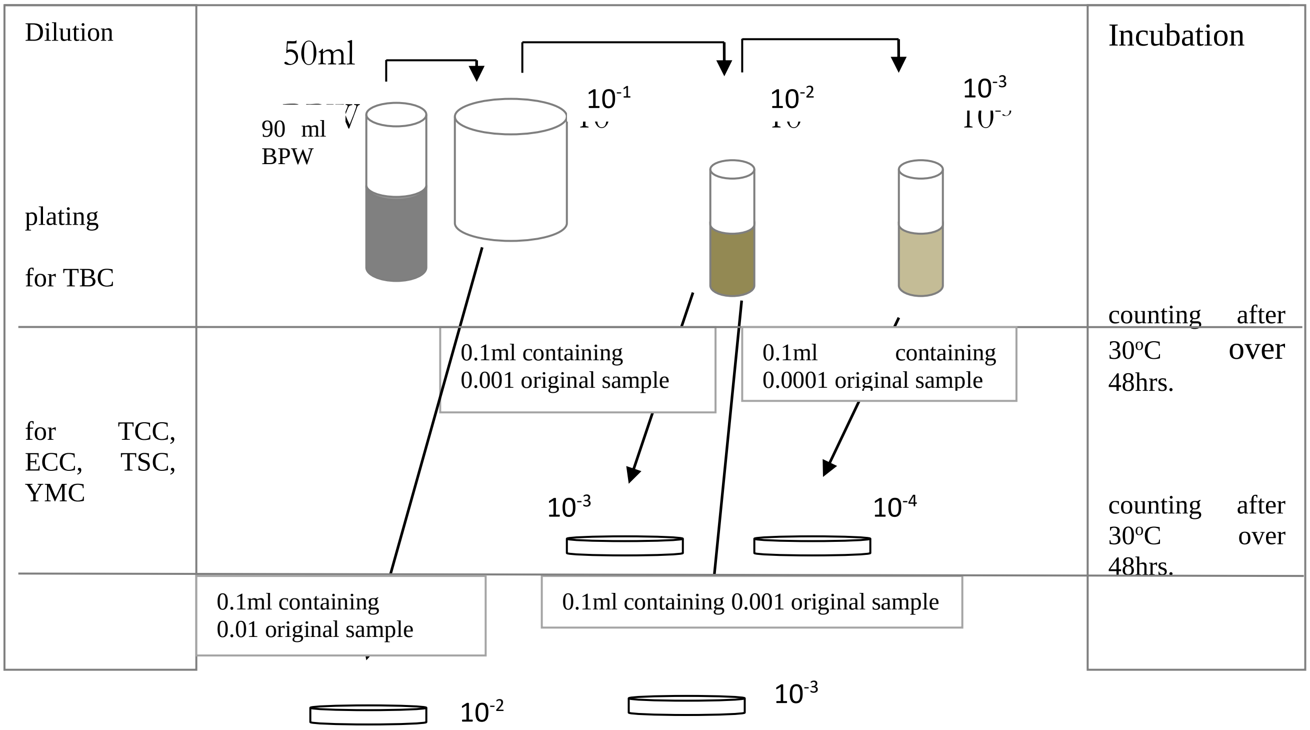
Annex 2. Age Determination protocol

At birth to 1 month	Two or more the temporary incisors teeth present. Within first month, entire **8** temporary incisors appear.
2 years	As long-yearling, the central pair of temporary teeth or pinches is replaced by the permanent incisors attain full development.
2-1/2 years	Permanent first intermediates. One on each side of the pinchers, are cut. Usually these are fully developed at **3** years.
3-1/2 years	The second intermediates or laterals are cut. They are on a level with the first intermediates and begin to wear at **4** years.
4-1/2 years	The corner teeth are replaced. At **5** years the animal usually has the full complement of incisors with the corner incisors show wear.
5-6 years	The permanent pinchers are leveled, both pairs of intermediates are partially leveled, and the corner incisors show wear.
7-10 years	At **7** or **8** years the pinchers show noticeable wear; at **8** or **9** years the middle pairs show noticeable wear; and at **10** years, the corner teeth show noticeable wear.
12 years	After the animal passed the 6th year, the arch gradually loses its rounded contour and becomes nearly straight by the **12th** year. In the meantime, the teeth gradually become triangular in shape, distinctly separated and show progressive wearing to stubs. These conditions become more marked with increasing age.

Annex 3. *Salmonella* Isolation Procedure

- **Non-selective pre-enrichment**
- ↓
- **25 g food in 225 ml of 10% buffered peptone water 37°C, 24 h**
- ↓
- **Selective enrichment**
- **0.1 ml in 10 ml Rappaport-Vassiliadis Soy Broth 42°C, 24 h**
- ↓
- **Isolation**
- **XLD with an inoculation loop ↙ ↘ BGA with an inoculation loop**
- **37°C, 24**
- **↘Streaking on nutrient agar ↙**
- ↓
- **Biochemical confirmation**
- ↓
- **TSI, LIA, SCA, UIA**

Annex 4. Procedure of *Salmonella* Enumeration in LNS



Annex 5. Laboratory Procedures for Salmonella

A. Xylose Lysine Deoxychoulat (XLD) Agar

- **Preparation**

- Suspend 56.85grams of the medium in one liter of distilled water. Heated with frequent agitation until a temperature of approximately 90ºC. Transfer immediately into a water bath at about 50ºC.
- Pour into Petri plates as soon as it has cooled. The medium should have a reddish color and be clear, or almost clear.

- **Test Procedure**

- Loop full of inoculums from RV were streaked on to pure XLD agar medium
- Incubate for 37^{0}C for 24hrs

- **Interpretations**

- Many cultures of *Salmonella* may produce colonies with large, glossy pink/red with black centers or may appear as almost completely black colonies.
- Transparent red (black center)

B. Triple Sugar Iron Agar

- **Preparation**
  - Mix and boil TSI with distilled water in ratio of 64.52g : 1 liter
  - Sterilized in 121^0 C for 15 min
  - Slant preparation
- **Test Procedure**

- Take one isolated colony from XLD/BGA and inoculate the middle and slant of the tube
- Incubate the medium for 18 hr at 37^{0}C
- Observe for color change and displacement of media

- **Interpretation**

- The amine production in the slant produces red color throughout the medium

- Production of insoluble black precipitate indication of H_2S production and
- H2S + ferrous sulfate à ferrous sulfide (Black precipitate)
- Cracking displacement of media due to gas production
- Yellow agar → acid production → sugar fermentation

C. **Citrate Utilization**

- **Preparation**

- Mix and boil Simmon's Citrate agar with distilled water in ratio of 24.28g : 1 liter
- Sterilized in 121^0 C for 15 min
- Slant preparation

- **Test Procedure**

- Inoculate Simmons Citrate Agar lightly on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old.
- Incubate at 35^oC to 37^oC for 18 to 24 hours. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium.
- Observe the development of blue color; denoting alkalinization.

Interpretation of Results

Citrate positive

Growth will be visible on the slant surface and the medium will be an intense Prussian blue. The alkaline carbonates and bicarbonates produced as by-products of citrate catabolism raise the pH of the medium to above 7.6, causing the bromothymol blue to change from the original green color to blue.

Citrate negative

Trace or no growth will be visible. No color change will occur; the medium will remain the deep forest green color of the uninoculated agar. Only bacteria that can utilize citrate as the sole carbon and energy source will be able to grow on the Simmons citrate medium, thus a citrate-negative test culture will be virtually indistinguishable from an uninoculated slant

D. Lysin Iron Agar

- **Preparations**

- ➢ Add lysine Iron agar to distilled water in the ratio of 34g : 1liter and boil with mixing
- ➢ Sterilized for 15 min at 121^0C
- ➢ Prepare slant

- **Test Procedure**

- ➢ Take one colony from pure culture and inoculate the medium and slant

- **Interpretation**

- ➢ Observe for color change to purple red in case of Salmonella positive

E. EB plate Count

- **Preparation**

- ➢ Add a plate count agar it distilled water in the ratio of 17.5g:1 liter
- ➢ Heat up to boiling point
- ➢ Sterilized in autoclave at 121^0C for 15min
- ➢ Poured in to the Petridish

- **Counting Procedure**

- ➢ Made dilution series prior to incubation
- ➢ Take 1ml from each dilution series and plate on the counting plate
- ➢ Incubate at 37^0C for 22-26hrs
- ➢ Count each distinct colony from minimum 30-300 CFU

Source: (Quinn *et al.* 1999)