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Evaluating the Effective Microbial Supplementation of Feed on the Load of *Salmonella* in Two Lymph Nodes of Beef Cattle in Eastern Ethiopia

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Abstract

The present study was conducted to evaluate the role of effective microbial supplementation to feed on the load of *Salmonella* in the mesenteric and sub-iliac lymph nodes of beef cattle. Bulls of Harer cattle breed managed at Chercher Oda-Bultum Farmers Union beef Farm were used as study subject. A total of 130 bulls were used using double blinded randomized controlled field trial based on parallel group design from January 2018 to July 2018. The study animals were randomly assigned to the treatment group (n = 100) and control group (n = 30). The feed of treatment group was mixed with EM at dose of 5×10^{10} cfu/day/head and supplemented for 90, 100 and 115 days while that of the control group was mixed with molasses, which acts as placebo. Both the treatment and control were slaughtered and two lymph nodes were collected from each animal under strict sterile condition and processed for the isolation and identification of *Salmonella* using standard procedure. A significant (p = 0.001) reduction in the load of *Salmonella* was observed in the lymph node of treatment group as compared to the control group. The load of *Salmonella* was significantly affected by length of feeding period and age of bulls. This study indicated that effective microbial supplementation to bulls from Harar cattle reduces the load of *Salmonella* in the lymph node of beef cattle thereby potentially minimizing the economic and public health impacts of *Salmonella* infection.

Keywords

Salmonella, Lymph Node, Effective Microbial, Load

1. Introduction

Gradual increase in world population and change in lifestyles have resulted in demands for quality oriented foods of animal origin. Meanwhile, the number of incidences of food poisoning cases is increasing throughout the world. On the 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 [1]. In this regard, many emerging and re-emerging pathogens associated with fresh or raw meat can be mentioned including *Salmonella* [2].

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 [3]. In Ethiopia, like other developing countries, it is difficult to evaluate the burden of food-borne pathogens. This is 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 [4] [5]. The widespread habit of raw beef consumption is a possible potential cause for the spread of food-borne illnesses in Ethiopia [5].

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 [6]. 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 [7] [8] [9]. These and related issues rise 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 is 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 [10]. Current estimates indicate that exposure to non-typhoidal *Salmonella* results in 93.76 million GIT illnesses and 155,000 deaths worldwide each year [11].

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 [12]. 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% *Salmonella* prevalence from water, meat transporting track and raw beef from butcheries respectively [12]. Approximately, the same rates were reported in the same or related chain of beef in some other studies in Ethiopia [13] [14] [15].

The above paragraph implies 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 means of *Salmonella* load reduction in lymph nodes for prevention and control.

To reduce the public health risk, clearly more needs to be done in *Salmonella* prevention. Therefore, the purpose of this study was to evaluate the effect of supplementing Effective Microbial (EM) in reducing the load of pathogenic *Salmonella* in lymph node of Harar cattle thereby safe beef provided for market.

2. Materials and Methodology

2.1. Description of the Study Area

The study was carried out at Charcher Oda Bultum Farmers Cooperative Union farm which is located in Oda Bultum district of Western Harerghea Zone, Oromia Regional State. The district is located at approximately 375 km east of Addis Ababa. Geographically this area has an altitude of 1400 - 3100 m.a.s.l and the specific location of the site is provided bellow (**Figure 1**). The area has a mean temperature ranging from 22˚C - 28˚C. It receives an average annual rainfall of

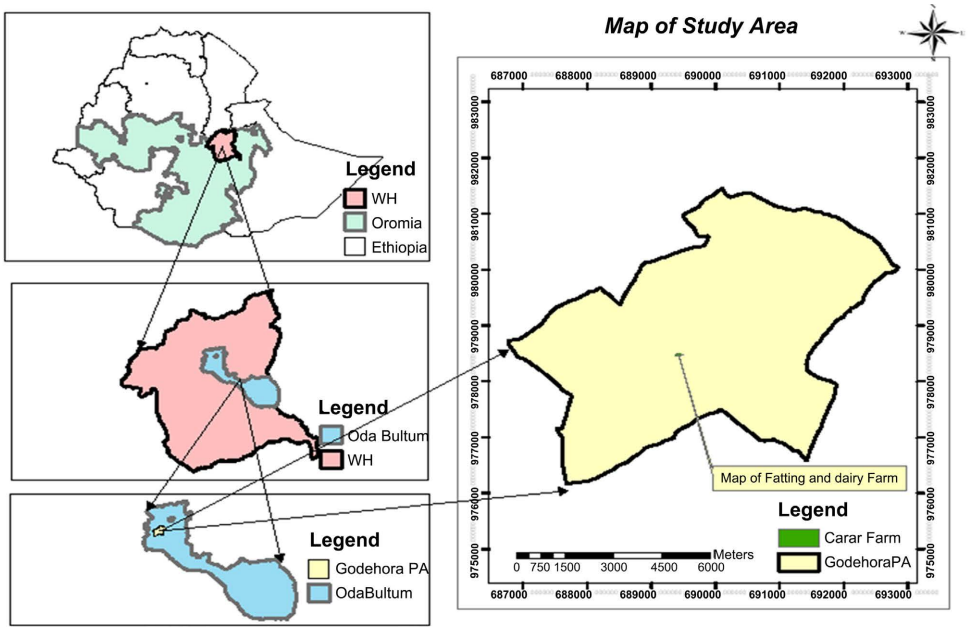

Figure 1. The study site location in Ethiopia.

900 mm - 1200 mm 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 [16]. The capacity of the farm can accommodate about 500 bulls for fattening and 250 dairy cattle with the objectives of beef and milk supply to the central market and local community. 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.

2.2. Study Animals

A total of 130 bulls from Harar cattle breed aged between 2 and 4.5 years managed in two pens of 100 (treated) and 30 (control) used for the study. All the bulls of study subjects were zebu breed of those mainly produced by the local small holders. Bulls were bought from the local markets Baddessa which is highlands and lowland areas Boke, Gabiba and Milkae. The production system in low land is mainly pastoral while in highland areas cattle are managed by thetering and supplied feed mainly by cut and carry system [17]. The Chercher Oda-Bultum Farmers Union purchased these bulls from smallholders and finished them in the feedlot to sell bulls at good body condition to markets at Addis Ababa, Mojo, Adama and institutes including Haramaya and Oda-Bultum Universities. Experimental bulls those slaughtered at Haramaya University Abattoir were sampled and examined at Haramaya University Microbiological laboratory.

2.3. The Study Animals Management

Body condition scoring [18] and age determination of the study animals were conducted according to the standards developed by Canadian Food Inspection Agency [19]. 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 in Ethiopia. The product was supplied in the form of solution which contained a mixture of EM, molasses and warm water (chlorine free) in the ratio of 1:1:18 liter. The product was feed mixed, according to manufacturer's recommendation, with the target dose being 5×10^{10} cfu/day/head of *Lactobacillus* bacteria [20]. Experimental bulls were fed for 90 days ($n = 42$), 100 days ($n = 40$) and 115 days ($n = 48$) days, then based on batch of animals were slaughtered. Treatment and control diets were administered for the duration of the feeding periods and separate feeding trucks were used for the two groups to administer the two different diets. All experimental bulls were provided similar basic diet such as grass, hay, teff straw, coffee hask and wheat

brand while treatment group were provided with EM.

2.4. The Study Design and Sampling Method

All animals were tagged and registered for onset of the experiment. For these purpose, animals' attendants and employed workers were trained on how to prepare, mix EM-microbial inoculants and feed to animals. Double blinded parallel-group-designed and randomized controlled field trial (RCFT) were conducted in which the treatment EM were supplemented to treatment group (n = 100) and control (n = 30) animals in each pen. Within pens the animals were clustered based on their biological differences considering animal age determination [19], body condition [18], body weight, sources and exposure time.

At the end of experimental period, experimental animals were slaughtered at HU abattoir. In both cohorts of the study, a sample of SLN and a sample of MLN were collected per carcass from both treatment and control groups immediately after slaughter. A total of 260 LNs (130 from SLNs and 130 from MLNs) were collected from all experimental bulls. Thus, pair of samples (SLN and MLN) from each experimental animal was collected aseptically and separately.

2.5. Sample Size Determination

The prospective randomized control field trial in parallel-groups-designed study based on feed supplemented by EM and non-EM supplemented group. Sample size was calculated by using the formula given by [21], which is appropriate in comparison of effects.

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

where: N = sample size, SE (standard error) = 5%; Hence, the required sample sizes were (n = 100) for treatment and (n = 30) animals for control cohort. Assuming that, confidence interval (CI) = 95%; desired absolute precision (a) = 0.05; Power (P) = 96%.

2.6. Sample Collection

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. A total of 84, 80 and 96 samples were collected separately from experimental bulls fed for 90, 100 and 115 days respectively. The samples were transported to Veterinary Microbiology Laboratory, College of Veterinary Medicine of 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.

2.7. *Salmonella* Enumeration

Quantitative culture methods were conducted according to [22], where one ml

of the Triptone Soya Broth (TSB) /LNs 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. EB plates were then held at 4˚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. The load of *Salmonella* was reported on a cfu/25g of lymph node basis.

2.8. Data Analysis

Raw data were interred to Microsoft Excel 2007© and analyzed using STATA 12.1. The *Salmonella* load data were transformed to $\log_{10}$ 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). Mixed effect Poisson regression and 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 considered as significant association.

3. Result and Discussions

3.1. Load of *Salmonella* in Lymph Nodes of the Study Animals

The study demonstrated a shift in load of *Salmonella* in LNs due to the influence of supplementation EM. Higher load of *Salmonella* was 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 of (EM), with the mean load of 2.14log ± 1.8 (t = 6.35; p = 0.000; 95% CI = 1.97 - 3.85) in MLNs. Whereas 1.29log ± 1.8 (t = 3.46; p = 0.0004; 95% CI = 0.55 - 2.04) difference were observed in EM treated animals in SLNs (Table 1).

3.1.1. The Load of *Salmonella* in Mesenteric Lymph Node (MLN)

Significant interaction was observed across three categories of days between load and slaughter day on a cfu/25g of lymph node basis with the mean difference of (1.64log ± 1.9; 95% CI = 0.26, 3.02; t = 2.40; p = 0.01), (2.22 ± 1.9; 95% CI = 0.91, 3.53, t = 3.44, p = 0.001), (0.7 ± 1.6; 95% CI = 1.7, 3.38, t = 6.09, p = 0.000) on the 90th day, 100th day and 115th days respectively (see Table 2). Among all considered risk factors pen (treatment), time (duration of treatment), and age of animals reveals significant difference in reduction of *Salmonella* load (see Table 3). The interaction of *Lactobacillus acidophilus* with pathogenic bacteria specific to MLN was not well documented. This direct us to former hypotheses on potential

Table 1. The total *Salmonella* mean Log_{10} cfu/25g difference among the study groups (pen).

Treatment and Types of LNs*	X ± SD	t-value	p-value	95% CI
Mesenteric LNs*				
Load of *Salmonella* in Control	2.78 ± 2.2	6.35	0.000	1.97 - 3.58
Load of *Salmonella* in Treatment	0.64 ± 1.4			0.36 - 0.92
Mean Load Difference	**2.14 ± 1.8**			1.47 - 2.80
Subiliac LNs*				
Load of *Salmonella* in Control	2.26 ± 2.1	3.46	0.0004	1.46 - 3.07
Load of *Salmonella* in Treatment	0.97 ± 1.6			0.63 - 1.29
Mean Load Difference	**1.29 ± 1.8**			0.55 - 2.04

Table 2. Effect feeding EM on *Salmonella* reduction in mean Log_{10} cfu/25g across time in mesenteric LN.

	90 days	100 days	115 Days
Mean Load of *Salmonella* (Control)	2.78 ± 2.08 [1.17 - 4.38]	2.96 ± 2.2 [1.25 - 4.67]	2.64 ± 2.3 [1.15 - 4.12]
Mean Load of *Salmonella* (Treatment)	1.13 ± 1.7 [0.51 - 1.75]	0.74 ± 1.5 [0.17 - 1.30]	0.09 ± 0.5[−0.1 - 0.3]
Mean Difference of *Salmonella* *CFU/LN	1.64 ± 1.9 [0.26 - 3.02]	2.22 ± 1.9 [0.91 - 3.53]	0.7 ± 1.6 [0.2 - 1.2]
t-Value	2.40	3.44	6.09
p = Value	0.011	0.000	0.000

*CFU = Colony Forming Unit, L*N* = Lymph Node.

Table 3. The *Salmonella* mean Log_{10} cfu*/node deference across risk factors for MLNs** in mixed effect.

	Mean Difference	Z-value	P > |Z|	95% CI	Wald test
Pen	1.34 ± 0.18	−7.52	0	0.99 - 1.69	**111.8**
Age	0.11 ± 0.02	5.44	0	0.071 - 0.151	
Body Condition	0.013 ± 0.028	−0.49	0.627	0.067 - 0.041	
Source	0.19 ± 0.195	−0.97	0.332	0.19 - 0.57	
Weight	0.033 ± 0.021	−1.57	0.117	0.008 - 0.075	
Time	0.093 ± 0.02	−4.6	0	0.53 - 0.133	

*Colony Forming Unit; **Mesenteric Lymph Node.

modes of action for *Lactobacillus* including production of antimicrobial compounds [23], reduction of gut pH by stimulating the lactic acid producing microflora [24], competition for binding of receptor sites that pathogens occupy [25], stimulation of immunomodulatory cells [26]. [27] supports 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 [26]. [28] 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 [29], 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.

3.1.2. The Load of *Salmonella* in Sub-iliac Lymph Node (SLN)

The *Salmonella* load reduction by 1.34log$_{10}$ cfu/25g in SLN was attributable to the supplementation of EM to the diet of beef cattle (see **Table 1**). 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 (0.96 ± 0.73; 95% CI = 0.51, 2.44; t = 1.32 and p = 0.09), (1.75 ± 0.70, 95% CI = 0.33, 3.17; t = 2.5; p = 0.008) and (1.28 ± 0.53, 95% CI = 0.21, 2.35; t = 2.41 p = 0.009) log$_{10}$ cfu/25g lymph node respectively (see **Table 4**). Among all considered risk factors pen (treatment) and time (slaughter days), reveals significant difference in reduction of *Salmonella* load (see **Table 5**). The trend in reduction of *Salmonella in* log$_{10}$ in the current study is in agreement with [30] reported 2.78 log$_{10}$ in USA of course the only published document in this regard up to the point of organizing this manuscript. Beyond the reduction trend, for the magnitude

Table 4. Effect feeding EM on *Salmonella* reduction in log cfu/25g across time in sub-iliac LNs.

	90 days	100 days	115 Days
Mean Load of *Salmonella* (Control)	2.34 ± 2.2 [0.63 - 4.05]	2.79 ± 2.1 [1.17 - 4.39]	1.80 ± 2.2 [0.39 - 3.22]
Mean Load of *Salmonella* (Treatment)	1.38 ± 1.8 [0.72 - 2.04]	1.03 ± 1.8 [0.38 - 1.69]	0.52 ± 1.3 [0.08 - 0.97]
Mean Difference of *Salmonella* CFU/LN	0.96 ± 1.9 [−0.51 - 2.44]	1.75 ± 1.9 [0.33 - 3.17]	1.28 ± 1.6 [0.21 - 2.34]
t-Value	**1.32**	**2.49**	**2.41**
p = Value	**0.097**	**0.01**	**0.009**

Table 5. The *Salmonella* mean Log$_{10}$ cfu/node difference across risk factors for SLN in mixed effect.

	Mean Difference	Z-value	P > |Z|	95% CI	Wald test
Pen	0.80 ± 0.17	−5.00	0.000	0.475 - 1.13	**54.43**
Age	0.040 ± 0.22	1.90	0.066	0.003 - 0.084	
Body Condition	0.083 ± 0.037	−2.27	0.025	0.011 - 0.156	
Source	0.196 ± 0.178	−1.15	0.27	0.152 - 0.544	
Weight	0.036 ± 0.019	−1.78	0.069	0.003 - 0.074	
Time	0.064 ± 0.018	−3.47	0.000	0.03 - 0.098	

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.

4. Conclusions and Recommendations

The result of this preliminary study demonstrated that effective microbial supplement in the diet of beef cattle reduced the load of *Salmonella* in sub-iliac and mesenteric lymph nodes significantly. Thus, result of this study showed the potential of effective microbial supplement in minimizing the contamination of beef with *Salmonella* organism. However, additional data should be generated to substantiate the result of this study before effective microbial supplement is recommended for wider use.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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