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Prevalence of Salmonella Species in Milk and Dairy Products in Major Milk Shade of Oromia, Ethiopia

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Abstract
Background: Foods of animal origin are major vehicles of *Salmonella* infections and a serious public health problem with increasing concern in the world, particularly for developing countries. More recently review studies on the prevalence of *Salmonella spp.* in dairy products demonstrate, it has been a median of 6% in raw milk and dairy products. However, almost all prior works in this area are limited to biochemical confirmation of suspected *Salmonella spp.* isolates from dairy a product that has high uncertainty to confirm. Due to this almost all report of the prevalence of *Salmonella spp*. in dairy products as well as raw milk was highly varied. Besides, almost all prior report in the prevalence of *Salmonella spp.* in dairy product in Ethiopia takes no consideration of the dairy value chain to identify major point of contamination of the product. To overcome the shortcomings of previous studies molecular techniques as well as milk and dairy value chain in the country were used to confirm the prevalence of *Salmonella spp*. in each value chain. **Methods:** A cross-sectional study was conducted on milk and dairy products in Welmera, Bishoftu, Asella, and Fiche milk shades of the Oromia region of Ethiopia from December to March 2020 to determine the prevalence and of *Salmonella spp.* A total of 480 samples of dairy products (192 raw milk from producers and collector, 192 pasteurized milk from processer and retailer, and 96 cottage cheese from producer and retailer value chain) were collected using simple random techniques from producers, collectors, processors, and retailer value chains. The samples were tested for Salmonella using Iso 6579-1: 2008 methods. The risk factor for contamination of these dairy products across the dairy value-chain was done using pre-tested questionnaires.**Results:** Among 480 samples 71 samples were confirmed for *Salmonella spp.* using latex agglutination test and molecularly by confirming the presence *highly conserved region of the invA gene.* The overall prevalence of 14.79% (71/480) 21.35% raw milk, 12.5% pasteurized milk, and 6.5% cottage cheeses were recorded from the total tested samples. Among dairy products in the study area; raw milk was the highest *Salmonella* prevalence followed by Pasteurized milk. In the dairy value chain, the highest prevalence of 62.5% was observed in raw milk from Bishoftu fo1lowed by 54.17% of pasteurized milk in Fiche. The prevalence of Salmonella among the study area was 9.6% (8/120), in Welmera, 33.6% (28/120), in Bishoftu, 16.8% (14/120) in Asella, and 25.2% (21/120) in Fiche.**Conclusions:** The result of this study indicates the safety of milk and other dairy product in the region were substandard of East African standard. Therefore, strict hygienic approaches and quality control measures should be applied to improve the safety of the products. Awareness creation should be required for producers, retailers, processors, and consumers regarding the quality and safety of milk and other dairy products.
Keywords: Prevalence, *Salmonella spp*, Dairy product
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Introduction

Foodborne diseases are a serious public health burden and massive economic losses in developed and developing countries. According to a World Health Organization report annually, 30% of the population in the developed country suffers from foodborne disease and up to 2 million deaths in developing countries (WHO, 2007; Mama & Alemu, 2016). In developing countries, most foodborne disease outbreaks are not reported or underreported (Odeyemi, 2016). The safety of foodborne diseases in dairy products is a major global concern especially for developing countries where the production of milk and dairy products done under poor hygienic, sanitary, and Agricultural practices (Karshima et al., 2013). The problem is more severe in countries like Ethiopia where there is a lack of food safety measures, and no reliable statistics on foodborne diseases due to poor reporting systems (Mukhopadhyay et al., 2012; Mama & Alemu, 2016). Milk has been considered as perfect food due to its

essential nutrients content required by the body in appropriate proportions. So it is the best medium for pathogenic microorganisms and potential vehicles for transmitting these pathogens to humans.

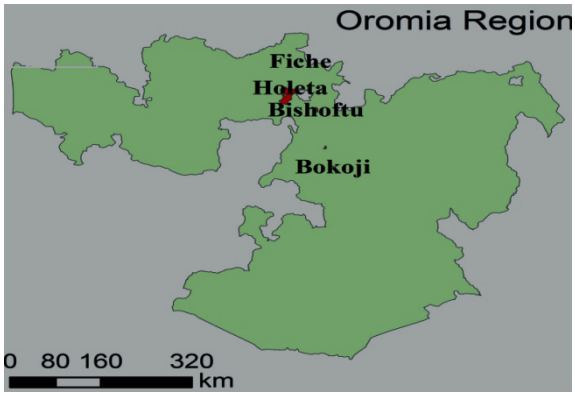
In Ethiopia, annually more than 3.3 billion liters of milk was produced from dairy cow in rural, urban, and pre-urban and 98% of productions were from the rural dairy system, while 2% from pre-urban and urban of Ethiopia(Brandsma et al., 2012). From annual milk production around 92% were produced in four regions of (Oromia 44.4%, SSNNP 21.7%, Amhara19.4% and Tigrai 6.3%) Ethiopia(CSA, 2017). Among the most common foodborne pathogens, *Salmonella* is considered the most prevalent pathogen worldwide (Sanchez et al., 2007). It is the most frequently isolated foodborne pathogen and is predominantly found in poultry, milk, and other dairy products (Yalew ST, 2020). *Salmonella* is a global public health concern, accounting for more than 93.8 million foodborne illnesses, and 155,000 deaths per year worldwide (Majowicz et al., 2010; Wang et al., 2015) They are a significant economic impact in humans and animals, predominantly in developing countries (Ejo et al., 2016)**.** The genus *Salmonella* is a gram-negative, rod-shaped bacteria facultative anaerobe, a flagellated bacterium that belongs to the family of Enterobacteriaceae (Yalew ST, 2020; Hailu et al., 2015). Currently, around 2610 *Salmonella* serotypes have been identified and more than 1540 belong to *Salmonella enterica* subsp. *enterica*, which accounts for the majority of *Salmonella* infections in humans (Eng et al., 2015). Milk and **dairy products** are major sources of *Salmonella* infection particularly for consumers who preferred to consume raw milk and milk products. Contamination of milk and other products by *Salmonella* species can occur from production to consumption at different value chains (Oliver Al., 2005; Nada et al., 2012). Many factors such as improper hygienic conditions in the farm, improper food storage, poor personal hygiene practices, inadequate cooling, and reheating of food items are the sources of *Salmonella* infections (Ejo et al., 2016b; Karshima N., 2013).

Food processing can improve the safety of food for consumers (Wambui et al., 2019), however; consumers' attitudes are increasing for the consumption of foods that are not or minimally processed foods such as raw milk and dairy products made from raw milk (Verraes et al., 2015). In Ethiopia, the incidence of salmonellosis caused by the dairy product is unknown; the risk factors associated with the contamination of animal products are not described, and there have not been studies on the attribution of sources to human illnesses. However, there is widespread raw animal product consumption habit in a noteworthy segment of the population are suggestive of the risk of acquiring *Salmonella* from animal products. Therefore, quantitative estimates of S*almonella spp* in raw milk and other dairy products could enable us to recognize the level of contamination and the comparative importance of dairy products as potential sources of *Salmonella* infections to humans. Many studies were done on the prevalence of *Salmonella spp.* in dairy products in Ethiopia. However, almost all prior works in this area are limited to biochemical confirmation of suspected *Salmonella spp.* isolated from dairy products that have high uncertainty to confirm. Due to this almost all previous report on the prevalence of *Salmonella spp*. in dairy products and raw milk was highly varied. Besides, almost all previous report in the prevalence of *Salmonella spp.* in the dairy product in Ethiopia takes no consideration of the dairy value chain to identify the major point of contamination of the product. To overcome the shortcomings of previous studies molecular techniques, as well as dairy value chains in the country, were used to confirm the prevalence of *Salmonella spp*. along the value chains. So that, this study is needed to estimate the prevalence of *Salmonella* species in milk and dairy product sources at different value chains using molecular detections of the isolates to know the accurate prevalence of *Salmonella spp*. in dairy products to intervene and improve the safety of milk and other dairy products in the country.

Assessment of risk factors associated with milk production systems is essential to ensuring the quality and safety of milk and milk products. This study was also aimed at identifying major risk factors for contamination of milk and milk products with Salmonella spp. across the dairy value chain in the study area

Materials and Methods

Study area



The study was conducted in four towns of Oromia regions namely Welmera which is located 49km from Addis Ababa to the west, Fiche located at 109 km from Addis Ababa to the northwest, Bishoftu located at 49 km to the east of Addis Ababa, and Asella which is located at 120 km to the southeast of Addis Ababa, these towns were selected based on their milk production potential in the region.

Study Design and Sampling.
A cross-sectional study was conducted between December 2020 and March 2020 to assess the prevalence of *Salmonella spp* in raw milk and other dairy products. The milk value chains involved in this study were milk producers (farmers), collectors, and retailers. A total of 192 raw milk samples were collected from a producer (n=96), collectors (n=96) and a total of 192 pasteurized milk were collected from a processor (n= 96) and retailers (n=96) whereas for cottage cheese 92 samples from two value chain producer (n=48) and retailers (n=48) were collected using simple random sampling techniques. Approximately 250 mL of raw and pasteurized milk was aseptically sampled into a sterile polyethylene bottle and 500gm of cottage cheese were collected in a sterile polyethylene zip bag and stored in a jet cooler less than 4 °C and transported to Holeta National Agricultural Biotechnology, Microbial biotechnology Research Laboratory and stored at below 4ºC until analyzed. The analyses of samples were done within 6-12 hours after delivered to the laboratory.

Microbiological analysis
The isolation and identification of *Salmonella spp*. were done according to ISO 6579-1: (ISO 2018). 25 mL/gm of the samples were transferred to 225 mL sterile buffered peptone water (Oxoid, CM 0509), and mixed well in a stomacher bag and incubated at 35 ºC for 18 hours then, 0.1 mL and 1ml of buffered peptone water cultured broth was aseptically inoculated into 10 mL of sterile Rappaport Vassiliadis (RV) broth (HIMIDIA) and Muller Kaufmann Tetrathionate broth (HIMIDIA) respectively. The culture in RVS broth was incubated at 41 ^{0}C for 24 hrs. And these in Muller Kaufmann Tetrathionate broth were incubated at 37 ^{0}C for 24 hrs. A 10µl loopful of cultured RV broth and Muller Kaufmann Tetrathionate broth was streaked onto both Xylose Lysine Deoxycholate (XLD) Agar (HIMIDIA) and Hektoen enteric agar (HIMIDIA), then the plates were incubated aerobically at 37 ºC for 24hrs. The suspected colonies (pink colonies or red colonies with or without black centers on XLD and blue-green to blue colonies with or without black centers on Hektoen enteric agar were picked up and sub-cultured on BHI agar (BBL^{TM}) and incubated at 35° ± 2.0° C for 24 ± 2.0 hours for molecular identification.

Latex agglutination test
Presumptive positive *Salmonella* colonies were confirmed using the latex agglutination test to identifying the motility of the isolates. One drop of saline was placed on to a well of the reaction card and a typical suspected colony was emulsified in the drop of saline and one drop of test latex (Oxoid, R6248pw, UK) was added and mixed with a sterile mixing stick for two minute and agglutination was examined. These forming agglutinations were indicative of *Salmonella spp*. positive *(PHE, 2018; El-baz et al., 2017).*

Molecular identification:
These culturally suspected Salmonella spp. isolates was confirmed by PCR based on the presence of the invA gene. The extraction of DNA of suspected isolates was performed by thermal cell lysis (El-baz et al., 2017; Reischl & Wittwer, 2001). The DNA was used as a template for the amplification of the highly conserved region of the invA gene using the primers Salm3 (*5´-* GCTGCGCGCGAACGGCGAAG-3') and Salm4 (*5´-* TCCCGGCAGAGTTCCCATT-*3´) which amplify a 389 bp fragment of the conserved invA gene sequence of Salmonella spp. Cycling conditions were optimized at* initial denaturation at 95°C for 5 min followed by 35 cycles of amplification (denaturation at 95°C for 90 s, annealing at 60°C for 60 s, and extension at 72°C for 90 s) using *by a thermocycler (BIO-RAD T100^{TM} Thermal cycler 621BR43010, Singapore)*, ending with a final extension at 72°C for 7 min. *The final PCR products were electrophoresed in 1.5% agarose gel stained with gel loading dye of 10% (B7025S, New England) using 100 bp DNA Ladder (Product no. N32315). The DNA band was visualized by gel documentation system (El-baz et al., 2017).*

Questionnaire
A total of 202 individuals 97 milk retailers, 7 milk processing factories, 40 cottage cheese retailers, and 48 cottage cheese farm markets were interviewed using a structured and pretested questionnaire developed with Kobo Toolbox. The questionnaires were orally translated to the local language (Oromifa/ Amharic) as needed during the interview. Validation of the questionnaire was made during the mock sampling study period involving all four study areas (Oromia, Amhara, SNNP, and Tigray).

Statistical analysis
Data obtained from the Laboratory test were analyzed using descriptive statistics of frequency distribution and percentage using SPSS version 25.0 Software (SPSS, 2017).

Results and Discussion
Salmonella is the most prevalent foodborne pathogens and important zoonotic microorganisms of economic

significance in humans, particularly in developing countries (Ejo et al., 2016). *In this study, from 480 samples (192 raw milk, 192 pasteurized milk, and 96 Cottage cheese) examined 14.79% (71/480) were found to be Salmonella spp. positive. This is comparable to 14.3% and 15% of Salmonella spp. prevalence in dairy products reported by (Beyene et al., 2016 and El-baz et al., 2017) respectively. From the overall dairy product prevalence of 14.79% (71/480); allotment of raw milk, Pasteurized milk, and Cottage cheese had 21.35% (41/192), 12.5% (24/192), and 6.25% (6/96) respectively. Among these dairy products relatively higher prevalence of Salmonella spp. were shown in raw milk followed by pasteurized milk (**Figure 1**). This higher Salmonella prevalence might be due to different factors like hygienic practice, storage, farm management, farm size, environmental factors, and season. The prevalence of Salmonella detected in raw milk in this study agreed with the work of (Gwida et al., 2014) who reported Salmonella in raw milk with a prevalence of 21% in Egypt. In contrast to this, higher Salmonella prevalence of 35.71%, 28.6% in Ethiopia, and 44.44% in* Egyptian dairy products was reported by Teshome et al., 2012; Addis et al., 2011, and Mahmoud et al., 2019 respectively. There is also a low prevalence of 3.1%, 6%, and 10.76% in raw milk was reported by *(Liyuwork et al., 2013, Ejo et al., 2016b and Tadesse & Gebremedhin, 2017, respectively in Ethiopia.*

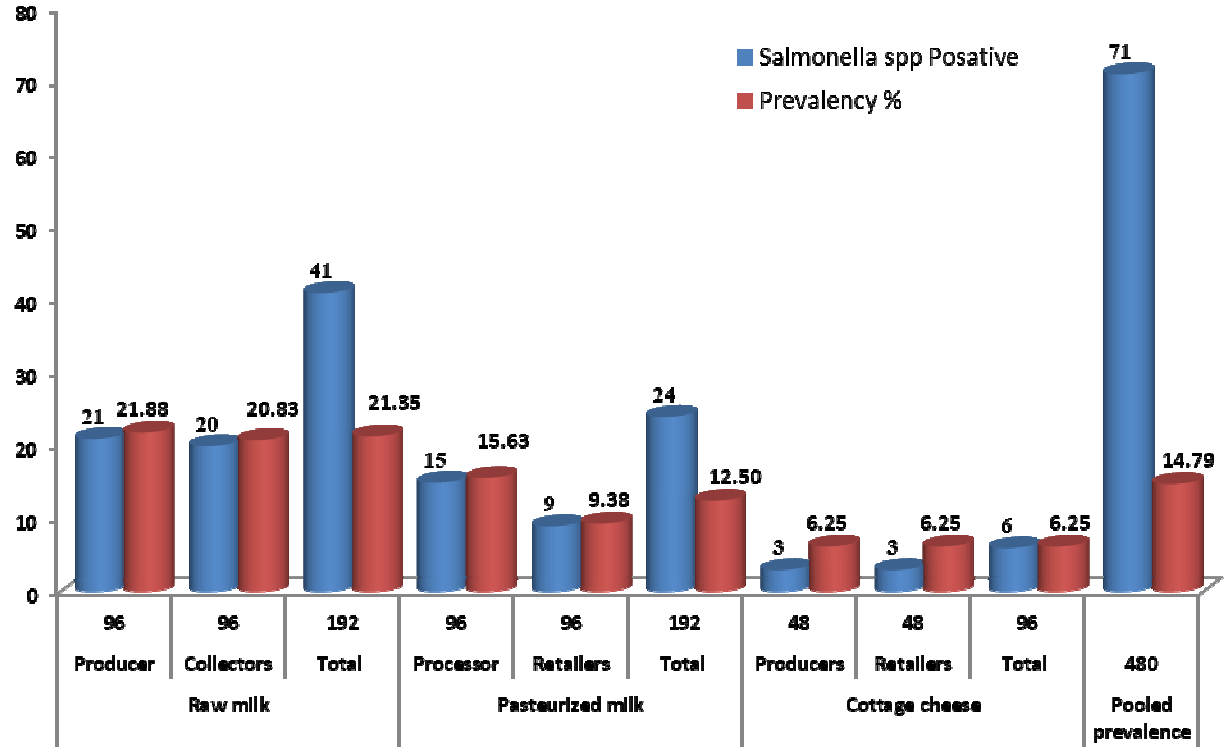


Figure 1: Prevalence of *Salmonella spp*. in milk and milk products across the value chain.

This variation in the prevalence of *Salmonella spp*. in raw milk among these studies might be associated with different factors like hygienic practices, farm management practices, sample size, sampling season, farm size, and test methods used for detection. *Other dairy product embraced in this study was Cottage cheese which was a prevalence of 6.5% across a value chain of producer and retailers. The prevalence of Salmonella detected in Cottage cheese in this study was higher than 2.1% reported by Zewudu, 2004, and Liyuwork et al., 2013 in Ethiopia. This might be due to post contamination from water used for processing, hygiene of equipment used for storing, and personal hygiene and storage condition (Karshima et al., 2013; Ejo et al., 2016b).*

*This finding is in contrast to the rep*ort of Ejo et al., 2016a and Mahmoud et al., 2019 who report zero prevalence of Salmonella in cottage cheese in Ethiopia. Processing effect during the preparation of Cottage cheese (Smoking of equipment used for fermentation, low pH during fermentation, and heat treatment) can destroy or inactivate Salmonella spp. (Mogessie, 2002). In Ethiopia, cottage cheese was prepared by spontaneous fermentation of raw milk for 2-3 days in a container well smoked with olive-wood sticks at ambient temperature. which reduces the pH to 4.0 to 4.2 (Mogessie, 2006). Butter was removed from the fermented milk by churning and buttermilk left was heated at 40 – 70^{o}C until distinct curd mass (Cottage cheese) forms. Smoking of equipment used for fermentation, low pH during fermentation, heat treatment, and production of antimicrobial substance of fermenting microbes during fermentation of milk can reduce foodborne pathogens like *Salmonella spp.* in Cottage cheese(Mogessie, 1993b; Mogessie & Fekadu, 1993a; Mogessie, 2006; Anteneh et al., 2011).

The different reports confirmed that *Salmonella* can't withstand proper pasteurization of time-temperature combination. This is confirmed by the work of Ejo et al., (2016a) who reported zero prevalence of *Salmonella* in pasteurized milk from Gondar, Ethiopia. However, in this study unanticipated higher *Salmonella spp.* prevalence of 12.5% was observed. This might be due to improper pasteurization or post contamination during packing, storage, and transportation.

Table 1: Showed the prevalence of Salmonella spp, across the dairy value chain and study area. The prevalence of Salmonella spp. was 6.6%, (8/120), 23.33% (28/120), 11.67% (14/120), and 17.5% (21/120) in Welmera, Bishoftu, Asella, and Fiche town.

Table 1: Prevalence of Salmonella spp. across location and value chain

Location	Product type	Value chain	Total samples	Positive samples	Prevalence %
Welmera	Raw milk	Producer	24	4	4/24 (16.67)
		Collector	24	3	3/24 (12.5)
	Pasteurized milk	Processor	24	0	0
		Retailer	24	0	0
	Cottage cheese	Producer	12	1	1/12 (8.33)
		Retailer	12	0	0
Bishoftu	Raw milk	Producer	24	15	15/24 (62.5)
		Collector	24	11	11/24 (45.83)
	Pasteurized milk	Processor	24	2	2/24 (8.33)
		Retailer	24	0	0
	Cottage cheese	Producer	12	0	0
		Retailer	12	0	0
Asella	Raw milk	Producer	24	2	2/24 (8.33)
		Collector	24	6	6/24 (25)
	Pasteurized milk	Processor	24	0	0
		Retailer	24	4	4/24 (16.67)
	Cottage cheese	Producer	12	2	2/12 (16.67)
		Retailer	12	0	0
Fiche	Raw milk	Producer	24	0	0
		Collector	24	0	0
	Pasteurized milk	Processor	24	13	13/24 (54.17)
		Retailer	24	5	5/24 (20.83)
	Cottage cheese	Producer	12	0	0
		Retailer	12	3	3/12 (25)

The variation among study areas might be due to differences in hygienic practices, geography, farm management, and quality control system in the area. Among these study areas relatively higher Salmonella spp. prevalence of (54.17%) was observed in raw milk from Bishoftu followed by 14.58% from Welmera town. Improbably no Salmonella spp. was detected in raw milk samples collected from Fiche town. On the other way, pasteurized milk samples from Welmera had zero prevalence for Salmonella spp. Contrary, pasteurized milk samples collected from Fiche and Asella town had a high prevalence of 37.5% and 8.33% respectively. Salmonella prevalence of *18.5 30%, and 35.71% in Jimma Zone, 2.1% and 28.6, in Addis Ababa and*,14.3% in Asella town was reported by Teshome & Anbessa, 2012); Liyuwork et al., 2013 and Addis et al., 2011, and Beyene et al., 2016 respectively at different time. This indicates that the prevalence of Salmonella spp. is not consistent among location (Geography), dairy product, dairy farm size, and duration, or season.

Major risk factor for each milk and milk products value chain in the study area

Table 2: shows the major risk factor for each dairy value chain in the study area. Among 202 individual interviewed 100%(7/7), from milk processing, 4.12(4/96), from milk retailers, 75%(30/38) from cottage cheese retailers, and 8.33%(4/48), had taken basic food safety and quality training, and all the milk processing factory and 28.57(2/7) were no any food safety management system certified as well as no took any HACCP training these might be a major cause for the contamination and safety of milk. Even though all milk pasteurizing factories were used high-temperature short time of 72 ^{O}C for 15 seconds 14.29 % of the milk processing factory didn't check the efficiency of pasteurization. Among the milk retailers, only 17.53% maintained the temperature of milk during transportation from processing plants and 14.14% were more than one hour to transport.

Table 2a: Milk Processing risk factor for *Salmonella spp*. contamination in milk and milk products Oromia region of Ethiopia

Risk factors	Frequency	Percentage
Processing factory food safety management system certification		
No	7	100
Personnel in a factory training skills of any basic food safety		
Yes	7	100
Processing plant personnel trained or took HACCP training		
Yes	5	71.43
No	2	28.57
Means of transport to deliver milk to the processing plant		
Four-wheel drive	5	71.43
Public transport	1	14.29
Maintain the temperature of milk during transportation		
No	4	57.14
Yes	3	42.86
Cooling system during transportation		
Yes	3	42.86
No	4	56.14
Frequency of cleaning processing plant		
Twice in a day	5	71.43
Once in a day	1	14.29
Sources of water used for cleaning		
Tap water	5	71.43
Groundwater	2	28.57
How often clean milk handling equipment		
Each time before and after handling	5	71.43
Twice in a day	1	14.29
Quality parameters checked during milk reception		
Water adulteration (Lactometer)	6	85.71
Acidity (Alcohol test)	6	85.71
Organoleptic (color, odor..)	5	71.43
Microbiological test	2	28.57
Time and temperature combination used for pasteurization		
HTST (High temperature short time) (72 °C for 15seconds)	7	100
The efficiency of pasteurization checking		
Yes	6	85.71
No	1	14.29

Table 2b: Milk retailer value chain risk factor for *Salmonella spp*. contamination in milk and milk products Oromia region of Ethiopia

Risk factor	Frequency	Percent
Any training related to safety and quality of milk		
No, attain any training	92	94.85
Got training	4	4.12
Means of transportation for delivering milk to the retail shop		
Cold trucks	17	17.53
Maintain the temperature of pasteurized milk during transportation		
yes	17	17.53
Time taken to deliver the milk to the retailer		
<30min	7	7.22
30min - 1 hr	6	6.19
>1hr	4	4.12
Packing materials		
Plastic pouches	95	97.94
Paper board packages	1	1.03
Storage of pasteurized milk until sell		
Fridge	96	98.97
Backup generator to be used in the case when electric power fails		
yes	11	11.34
no	77	79.38
Separate refrigerator to store pasteurized milk until sell		
Yes	78	80.41
No	18	18.56

Table 2c: Cottage cheese retailer's risk factor for *Salmonella spp*. contamination in milk and milk products Oromia region of Ethiopia

Risk factor	Frequency	Percentage
Any training related to safety and quality of cottage cheese handling		
Yes	30	75
No	8	20
Transportation from processor to retail point		
Trekking on foot	5	12.5
Public transport	14	35
Animal drawn cart	3	7.5
Refrigerated vehicles/ cold chain	13	32.5
Time taken to transport cottage cheese from processing facility to retail shop		
½ hr. – 1 hr.	19	47.5
Less than 30 min	14	35
Over 1 hr	5	12
Packaging material in which cottage cheese is received by retailer from farm market or processor		
Polyethylene plastic container "festal"	35	87.5
Plastic bucket	2	5
The quality of packaging materials		
Good (Maintains product hygiene, prevents food/cheese spoilage)	21	52.5
Poor (Porous, does not maintain the hygiene of cheese and exposes the cheese to contamination)	17	42.5
Backup generator to be used in the case when electric power is out		
Yes	19	47.5
No	17	42.5

Table 2d: Cottage cheese farm market risk factor for *Salmonella spp*. contamination in milk and milk products Oromia region of Ethiopia

Risk factor	Frequency	Percentage
Any training related to cottage cheese handling practices		
No	44	91.67
Yes	4	8.33
Cottage cheese preservation means until sale		
Placing in a whey	25	52.08
Placing in a vessel of cold water	10	20.83
Refrigerator	3	6.5
Packaging material for cottage cheese handling		
Plastic bucket	40	83.33
House paint buckets	2	4.17
Quality of packaging materials		
Poor (Porous, does not maintain the hygiene of cheese and exposes the cheese to contamination)	40	88.33
Good (Maintains product hygiene, prevents food/cheese spoilage)	3	6.25
Cottage cheese transportation to the farm market		
Trekking on foot	15	31.25
Public transport	13	27.08
Three-wheel drive "Bajaj"	9	18.75
Animal drawn cart	7	14.58
Motor bicycle	1	2.08
Time taken from household to farm market		
½ hr. – 1 hr.	18	37.5
Over 1 hr	14	29.17
Less than 30 min	11	22.92
Regularly cleaning of cottage cheese handling equipment		
Yes	47	97.92
No	1	2.08

More than 83% of the packing materials of cottage cheese from farm market/processer were plastic bucket and More than 42% of these packing materials were poor quality, which has porous, does not maintain the hygiene of cottage cheese which expose the cottage cheese for contamination (Table 2d).

Conclusions

The result in this study indicates the safety of milk and other dairy product in the region were substandard of East African standard. So, that to ensure the quality and safety of raw milk and milk products; training and awareness creation for every actor engaged in milk and dairy production (producers, collectors, processing factory, retailers/supermarkets) and public education regarding the hygienic practices, safety, and risks of consumption of raw or dairy products are important lines of defense against *Salmonella* infection and other food-borne pathogens transmitted through dairy products in the region.

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