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Total Bacterial Count and Identification of *Staphylococcus* species from Critical Control Points of Raw and Processed Milk in Selected Dairy Farm in Bishoftu Town, Ethiopia

Ararsa Kumala[1, 2], Fethu Lemma[1], Bruk Abraha Fitwi[1*]

[1]*Haramaya University, College of Veterinary Medicine, P.O. Box 138, Dire Dawa, Ethiopia.*
[2]*Alage Agricultural Technical Vocational Education and Training College, Ethiopia.*

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ABSTRACT

A cross-sectional study was carried out from November 2016 to May 2017 in Bishoftu town, Ethiopia to study total bacterial count (TBC) and detection of *Staphylococcus aureus* from critical control points (CCPs) at dairy farm (water, milker's hands, milking bucket, udder milk, milk storage, pooled milk, pasteurized milk and yoghurt). A total of 60 samples were subjected for plate count agar (to estimate the colony forming units (cfu) per ml), and bacteriological culture and biochemical tests for the detection of *S. aureus* and other gram-positive cocci. Descriptive statistics and analytic statistics such as one way ANOVA test was used to calculate the mean difference in cfu/ml among sample sources. The log10 cfu/ml of mean value of bacterial load were 6.10, 5.78, 5.35, 5.15, 4.75, 4.52, 4.42, and 4.32 for pooled milk, water, milker's hands, udder milk, milk storage, yoghurt, milking bucket, and pasteurized milk, respectively. Comparison of TBC from different sampling points indicated that pooled milk samples had significantly higher ($p<0.05$) bacterial load than other sampling points. Generally, raw milk had significantly higher ($p<0.05$) bacterial load ($5.63x10^5$ cfu/ml) as compared to the processed milk and contact materials. Out of the total 60 bacterial growth, *Staphylococcus* species accounts 73.3% (44/60) of the total growth, with coagulase negative staphylococci (CNS) and *Staphylococcus aureus* accounting for 36 (60.0%), and 8 (13.33%) of the isolates, respectively. *S. aureus* was isolated mainly from milker's hand, udder milk, and pooled milk samples. We found that the total bacterial count from contact surfaces, raw milk and dairy products was below the recommended standard and the presence of *Staphylococcus* isolates at different CCPs indicates poor milk production practices. The high level of contamination and presence of potentially pathogenic bacteria could pose public health risk due to infection and intoxications. Hence, the dairy farm should design a strategy to improve the hygienic practice on milk production, handling, and processing.

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Introduction

Milk is synthesized in specialized cells of the mammary gland and is virtually sterile when secreted into alveoli of the udder (Shunda *et al*., 2013). Milk produced by healthy udder contains a very few bacteria, which is about 500 to 1,000 colony forming units per ml of milk. However, the estimated number might increase due to contamination from external sources such as air, milker's hand, milking environment, and other extraneous substances (Pandey and Voskuil, 2011). In Ethiopia, the milking practices are different based on production systems. For instance, milking of cows under smallholder production system is generally poor, which is characterized by absence of udder washing and the use of calve suckling for prior to milking (Zelalem, 2012). In the highlands of Ethiopia, about 45% of small-scale milk producers do not have the facilities for pasteurize of milk and organoleptic properties of dairy products are the commonly used quality tests at the time of purchasing (Maria *et al*., 2010).

Milk-borne infections and intoxication are the most common threats for public health. This is especially true in developing countries where production of milk and milk products are taking place under poor sanitary conditions coupled with poor handling systems (Teshome, 2016). Milk may contain both pathogenic and apathogenic microorganisms (Solomon *et al*., 2014). The load of microbes in milk depends on the post-production handling practices as well as efforts made to prevent spoilage (Worku *et al*., 2012).

Total bacterial count (TBC) is the means by which the level of contamination of milk and milk products is evaluated and allows us to infer the likely adverse effects on industrial productivity and safety of milk (Gargouri *et al*., 2013). TBC doesn't tell us microbial populations in terms of pathogens and non-pathogens (Solomon *et al*., 2013). Despite the labor intensiveness and inaccuracy for high bacterial count, the standard plate count is generally accepted as the most accurate and informative method of testing bacteriological quality of milk (Yien, 2014). Based on the standards set by dairy regulations,

*Corresponding author: Bruk Abraha Fitwi
E-mail address: abraha.ashebr@yahoo.com

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raw and pasteurized milk should not be exceeded to contain 100,000 cfu/ml and 20,000 cfu/ml, respectively (PMO, 2009).

In Ethiopia, the increase in milk production has resulted in improvements of dairy cattle management techniques. For instance, controlling of bacterial contaminants from various contact surfaces has been practiced to improve the microbiological quality of milk on dairy farms (Zelalem, 2012). In Bishoftu town of central Ethiopia, despite the huge milk production potential, little is known about the bacterial load on different contact surfaces of milk production environments. In addition, limited data is available on the presence of common spoilage and pathogenic bacteria such as *Staphylococcus* species and other related bacteria along the milk production channels in dairy farms. Therefore, the study was conducted to estimate the bacterial load (total bacterial count) from CCPs along the milk production channels, and to investigate the occurrence of *S. aureus* at the selected sampling points in Bishoftu, Ethiopia.

Materials and methods

Description of the Study Area

Bishoftu, is located 45 km South-east of Addis Ababa on the highway of Adama town in Oromia National Regional State, Ethiopia. It is geographically found at 9 O N latitude and 40 O E longitude. Bishoftu is the center of Ada'a Liben Woreda and it has a total land area of about 1610.56 Km2 and is divided in to three agro-ecological zones namely midland (94%) highland (3%), and lowland (3%) and at an altitude of 1850 meters above sea level in the central high lands of Ethiopia. It experiences a bimodal pattern of rainfall with the main rainy season extending from June to September (of which 84% of rain is expected) and a short rainy season from March to May with an average annual rainfall of 800mm. The mean annual minimum and maximum temperatures are 12.3 and 27.7 OC, respectively, with an overall average of 18.7 OC. The highest temperatures are recorded in May and the mean relative humidity is 61.3% (CSA, 2008).

Study Design and Study Animals

A cross-sectional study was conducted from November 2016 to May 2017 to determine the total bacterial count and to identify *Staphylococcus aureus* and other *Staphylococcus* species at CCPs (washing water, milker's hands, milking buckets, milk from udder, milk storage, pooled milk, pasteurized milk and yoghurt) of milk production and processing. The target animals were lactating cows of Holstein Friesian (HF) breeds in the selected farm in Bishouftu. One large scale dairy farm was selected purposively due to the presence of advanced milk production and processing operations.

Sampling Method

A total of 60 samples were collected from different CCPs. All samples were collected aseptically and processed immediately as described by APHA (1992). About 20 ml of raw milk samples were collected from two critical control points (four quarters of cows' udder and from the milk storage) using screw capped universal bottles. Moreover, pasteurized milk, freshly prepared yoghurt, and water samples were sampled as indicated for sampling of milk from udder. Prior to milk sampling, swab samples (from milking equipment's, milk storages, and milker's hands) were collected after marking the contact surfaces with 5x10 cm template, ie, a cotton tip moistened with transport medium was rubbed over the marked area and transferred into sterile screw-capped universal bottles. All samples were labeled with permanent markers and transported to National Veterinary Institute Bacteriology Laboratory using a box containing an ice pack within 30 minutes of sample collection and kept in refrigerator at 4°C until subjected for bacterial culture.

Laboratory Analysis

Total Bacterial Count

One ml from each sample of raw milk, water, pasteurized milk, and yoghurt were transferred to test tube containing 9ml of sterile tryptone soya broth and thoroughly homogenized to give 1:10 dilution (first dilution). Samples collected by cotton swabs were premixed in zero dilution. Seven serial dilutions (10-7) were made by transferring 1ml of the previous dilution in 9ml of Tryptone Soya Broth. Then, 0.1 ml of each dilution was inoculated on Plate Count Agar (Oxoid, UK) in duplicate using pour on plate technique. The inoculated plates were incubated at 32 ^{O}C for 48 hours. Total bacterial count was done by counting grown colony on the plate with the range of 30 to 300 by using colony counter. The number of microorganisms (colony forming units) per milliliter of milk was calculated using the following mathematical formula as described by APHA (2012):

$N=\sum C/[(1\times n1)+(0.1\times n2)d]$

N = number of colonies per milliliter of milk

$\sum C$ = Sum of all colonies on all plates counted

n1 = Number of plates in first dilution counted

n2 = Number of plates in second dilution counted

d = dilution from which the first counts were obtained

The CFU/mL is then calculated by the simplified formula used by Solomon *et al*. (2013).

CFU/mL= average number of colonies from duplicated plates /Dilution factor x volume plated

Isolation and Identification of *Staphylococcus* species

Parallel to plate count method, samples were streaked on to mannitol salt agar (MSA) and incubated aerobically at 37 ^{O}C for 24 hours. Then both yellow and pink pigment producing colonies were suspected as *Staphylococcus aureus* and other Staphylococci species, respectively. Then the isolated colonies were transferred on to nutrient agar plate and incubated at 37 ^{O}C for 24hrs to get pure colonies. Then primary identification of suspected colonies was performed based on Gram's reaction, cellular morphology, catalase test, oxidase test, and oxidation fermentation (OF) test. The colonies which were gram-positive cocci in clusters, catalase positive, oxidase negative, and fermentative, hence considered as *Staphylococcus* species were subjected for tube coagulase test to identify *S. aureus* from coagulase negative *Staphylococcus* species as described by Quinn *et al*. (2002). Those yellow colonies on MSA and able to coagulate rabbit plasma were judged as *S. aureus*, while colonies unable to coagulate rabbit plasma were considered as coagulase negative Staphylococci (CNS).

Data Management and Analysis

The collected data were entered into Excel spreadsheet 2010. Total bacterial counts expressed as colony forming units (CFU/ml) was transformed into log10 prior to statistical analysis using SPSS version 20. Both descriptive and analytical statistical methods were applied. One way ANOVA test was used to calculate the mean difference among sample types. P-value less than 0.05 were considered statistically significant.

Results

Total Bacterial Count

In this study, a total of 60 samples from different sources (N=8) were subjected for total bacterial count (Table 1). The result revealed that the TBC ranged from 2.10 x10^4 to 1.26 x10^6 cfu/ml. The highest bacterial count was recorded from pooled milk sample (1.26 x10^6 cfu/ml), while the lowest value was recorded in pasteurized milk (2.10 x10^4 cfu/ml). Comparison of TBC from different sampling points indicated that pooled milk samples had significantly higher ($p<0.05$) bacterial load than other sampling points (Table 1).

On the other hand, analysis was conducted based on categorization of critical points in to three groups namely; raw milk, processed milk (pasteurized and yoghurt), and contact materials (water, milkers' hand, milking bucket, milk storage). Accordingly, the highest bacterial load was detected in raw milk (5.63x10^5 cfu/ml), followed by contact materials (2.01 x10^5 cfu/ml) and processed milk (2.57x10^4 cfu/ml) and the difference between raw milk and processed milk was statistically significant ($p<0.05$) (Table 2).

Overall Prevalence of Staphylococcus aureus

From the total samples examined (n=60), all showed bacteria growth. And out of the total 60 bacterial growth, 52 (86.67%) belongs to gram positive cocci and *Staphylococcus* species accounts 73.3% (44/60) of the total growth. Among the *Staphylococcus* species, coagulase negative staphylococci (CNS) and *Staphylococcus aureus* accounts for 36 (60.0%), and 8 (13.33%), respectively (Table 3).

Discussion

Hygienic handling of milk and dairy products along the various stages in the milk processing chain includes; the hygiene of cow's udder; the cleanliness of milker's hand and milking utensils; proper storage and transport of milk; and proper treatment/processing of raw milk (teGiffel, 2003). In order to provide safe and healthy milk products, the Hazard Analysis and Critical Control Points (HACCP) system should be implemented starting from milk collection, through processing and storage. Thus, microbial exposure assessments are critical components of the risk analysis (FAO, 2007).

In the present study, among the assessed critical control points, highest total bacterial count (TBC) was recorded in pooled milk samples (1.26 x10^6 cfu/ml; 6.10 $\log_{10}$ cfu/ml) followed by water samples (6.11 x10^5 cfu/ml; 5.79 $\log_{10}$ cfu/ml), milker's hand (2.27 x10^5 cfu/ml; 5.36 $\log_{10}$ cfu/ml) and udder milk (1.42 x10^5 cfu/ml; 5.15 $\log_{10}$ cfu/ml) while the least count was from pasteurized milk (2.10 x10^4cfu/ml; 4.32 $\log_{10}$ cfu/ml). The bacterial count from the pooled milk and udder milk is above the acceptable level of 1x10^5 bacteria per ml of raw cow's milk (O'Connor, 1994).

The current report of TBC from cow milk (1.42 x10^5 cfu/ml; 5.15 $\log_{10}$ cfu/ml) is slightly higher than reported by Fekadu (1994) from Southern Ethiopia (3.8-4.00 Log_{10} cfu/ml), while it is within the range reported by Abebe *et al.* (2012), from southern Ethiopia (4.57-9.82 $\log_{10}$ cfu/ml). However, previous research conducted in different part of the country revealed much higher microbial counts. Thus, Worku *et al.* (2012), Alganesh (2000), Tassew and Seifu (2011), Solomon *et al.* (2013), and Teshome *et al.* (2014) reported mean TBC ($\log_{10}$ cfu/ml)

Table 1. Total bacterial count from different sample sources of dairy farm

Sample type	Mean TBC (cfu/ml)	$\log_{10}$ cfu/ml	F-value (P-value)
Pooled milk (n=6)	1.26 x10^{6a}	6.102254	6.28 (0.000)
Water (n=6)	6.11x10^{5b}	5.786584	
Milker's hands (n=6)	2.27 x10^{5b}	5.357347	
Udder milk (n=10)	1.42 x10^{5b}	5.152983	
Milk storage (n=6)	5.70 x10^{4b}	4.756065	
Yoghurt (n=6)	3.34 x10^{4b}	4.524911	
Milking bucket (n=10)	2.63 x10^{4b}	4.420774	
Pasteurized milk (n=10)	2.10 x10^{4b}	4.322881	

Mean values with different letter varied significantly ($p<0.05$) TBC= Total bacterial count;

Table 2. Total bacterial count of from milk samples and contact surfaces

Sample type	Mean TBC (cfu/ml)	F-value (P-value)
Raw milk (n=16)	5.63x10^{5a}	4.251 (0.019)
Processed milk (n=16)	2.57x10^{4b}	
Contact materials (n=28)	2.01 x10^{5ab}	

Mean values with different letter varied significantly ($p<0.05$); TBC= Total bacterial count.

Table 3. Frequency and percentage of *Staphylococcus aureus* and other *Staphylococcus* species

Samples	Gram Positive Cocci	*Staphylococci* Species	Coagulase Negative *Staphylococci*	*S. aureus*
Water (n=6)	5	4 (66.6%)	4 (66.6%)	0 (0%)
Milker's hands(n=6)	6	6 (100%)	4 (66.6%)	2 (33.3%)
Milking bucket (n=10)	6	6 (60%)	5 (50%)	1 (10%)
Udder milk (n=10)	10	7 (70%)	5(50%)	2 (20%)
Milk storage (n=6)	6	5 (83.3%)	5 (83.3%)	0 (0%)
Pooled milk (n=6)	6	5 (83.3%)	2 (33.3%)	3 (50%)
Pasteurized milk (n=10)	8	7 (70%)	7 (60%)	0 (0 %)
Yoghurt (n=6)	5	4 (66.6%)	4 (66.6%)	0 (0%)
Total (n= 60)	52 (86.67%)	44 (73.3%)	36 (60%)	8 (13.3%)

of 7.36-7.88 in Borana; 7.87 in BilaSayo and GutoWayo district of Eastern Wolaita; 7.58 in Bahir Dar Zuria and Mecha district; 7.07 in Debre Zeit, and 7.125 in Shashemene towns, respectively.

The present finding of a total bacterial count from contact surfaces (4.42-5.36 $\log_{10}$ cfu/ml) is far lower than the earlier reports of 10.28 $\log_{10}$ cfu/ml from containers in Hawassa town (Haile *et al*., 2012), 9.10 $\log_{10}$ cfu/ml for milk samples collected from different parts of Ethiopia (Zelalem, 2012), and 9.137 $\log_{10}$ cfu/ml from vendors in Dire Dawa town (Teklemichael *et al*., 2013).

In the present study, the TBC from pasteurized milk (2.10 x10^4 cfu/ml; 4.32 $\log_{10}$ cfu/ml) and yoghurt (3.34 x10^4 cfu/ml; 4.52 $\log_{10}$ cfu/ml) scored higher counting of bacterial load when compare to other samples. This high level of contamination could arise from contaminated equipment and personnel; delayed pasteurization; and substandard heat treatments. Moreover, the nature of some organisms, especially the biofilm formation ability by most members of *Staphylococcus* species could confer survival in different contact surfaces; and helps to resist adverse conditions such as the acidic environment of yoghurt.

Milker's hands also presented having 2.27 x10^5 (5.36 $\log_{10}$) cfu/ml bacterial loads, which could be due to improper washing of hands (not using detergents) and also it could be from using contaminated water, as it was reported to have a bacterial load of 5.78 $\log_{10}$ cfu/ml. The study also showed that the bacterial load in milking bucket and storage were 4.402 and 4.75 $\log_{10}$ cfu/ml, respectively, which were lower compared with other critical control points. The source of contamination could be water used for washing of utensils, milker's hands, and resident bacteria of storage utensils.

The present study revealed the presence of *Staphylococcus* in 44 out of 60 (73.3%) analyzed samples. This indicates there is high contamination at each CCPs with *Staphylococcus* species as a result of poor hygiene from milking to processing. The detection was made in all proposed CCPs with different proportion, with the high proportion being in milker's hand (100%). The percentage of *Staphylococcus* species occurrence in raw milk in the present study was high compared with the finding of Debebe (2010) who identified 24.4% of *Staphylococcus* species from milk samples collected from milk producers and street-vendors in and around Addis Ababa city. There were 36 (60%) coagulase negative *Staphylococcus* species from CCPs which is comparable with the report of Bendahou *et al*. (2008) who documented coagulase-negative *Staphylococcus* to be the dominant (54%) in raw milk and milk products in North Morocco.

The finding of the present study also revealed that different CCPs were contaminated with pathogenic *S. aureus* at a range 0-50%. However, the overall prevalence of *S. aureus* (13.3%) is lower compared with the earlier finding of Shunda *et al*. (2013) in Mekelle town (44.4%) and Daka *et al*. (2012) in Hawassa town (48.7 %) of Ethiopia. The occurrence of *S. aureus* in dairy farm milk samples could be associated with poor udder preparation and poor hygiene during milking.

Conclusion

In the present study we observed that milk produced in the farm is of low quality when compared with standard bacterial load for raw and processed milk. Though there is significant difference in the bacterial load between raw and processed milk, the bacterial load in the processed milk (eg pasteurized milk and yoghurt) is still above the recommended level for human consumption. Furthermore, the occurrence of *S. aureus* and other Staphylococci in the samples from all CCPs reflects the poor hygienic practices during milking. The high bacterial loads and presence of pathogenic bacteria posed a public health hazard as well as affects milk processing qualities. Thus, implementation of hazard analysis critical control point is required at each critical point from milk production to processing.

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Conflict of interest

Authors declare that they have no conflict of interest.

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