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Bacteriological quality and antimicrobial resistance patterns of isolates from raw milk at selected dairy farms in Ethiopia.

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This is to certify that the thesis prepared by **Kaleab Sebsibe** entitled: "**Bacteriological Quality, and Antimicrobial drug resistance patterns of isolates from raw milk at selected dairy farms in Ethiopia**", and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Diagnostic, and Public Health Microbiology) complies with the regulations of the University, and meets the accepted standards with respect to originality, and quality.

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Abbreviations

AMR	Antimicrobial Resistance
ACC	Aerobic colony count
ATCC	American type culture collection
BGLB	Brilliant green bile lactose broth
BPW	Buffered peptone water
CLSI	Clinical Laboratory Standard Institute
CFU	Colony forming unit
EC	*E. coli* broth
EMB	Eosin methylene blue
ETB	Ethiopian Birr
EPHI	Ethiopian Public Health Institute
ESBL	Extended Spectrum beta-lactamase
GN	Gram negative
GP	Gram positive
KIA	Kligler iron agar
MCC	Milk collection center
ML	Milliliter
MDR	Multi-drug resistance
MSA	Mannitol salt agar
SD	Standard deviation
SOP	Standard operating procedure
SNNP	Southern Nations and Nationalities Peoples
SPSS	Statistical package for social sciences
TBC	Total bacterial count
TCC	Total coliform count
TSA	Tryptone soya agar
VRBA	Violet red bile lactose agar
WHO	World Health Organization

Abstract

Background: Food-borne illness affects up to 30% of the population in developing countries each year, with great impact on public health, and the economy. Milk-borne infections cause acute, and severe disease. However, in developing countries there is limited information regarding bacteriological status of raw milk. This study aimed to assess the bacteriological quality and antimicrobial resistance patterns of isolates from raw milk.

Objective: To evaluate the bacteriological quality and antimicrobial resistance patterns of isolates from raw milk at different dairy farms found in Ethiopia.

Methods: A cross-sectional study design was conducted from August 2022–April 2023.A total of 176 raw milks samples were selected using multi-stage sampling technique. Bacteriological tests such as aerobic colony count, total coliform, thermotolerant coliform, *Salmonella* and *Shigella* detection, and *Staphylococcus* enumerations were evaluated. VITEK-2 compact was used for further bacterial identification, while antimicrobial susceptibility test was determined by disc-diffusion techniques. SPSS V.27 was use for data analysis; descriptive statistics were used.

Result: The mean aerobic colony count, total coliform, thermo-tolerant coliform, *E. coli*, and *Staphylococcus* count was,3.2, 2.70, 2.69, 2.9, and 3.1 $\log_{10}$ cfu/ml respectively. *E. coli (77*%), *Klebsiella spp. (5%), Salmonella spp.* (1.4%), *S. aureus* (13.6%), *S. saprophyticus (9.8%)* were also isolated. Gram-negative isolates showed sensitivity for Meropenem (96.8%) and Ciprofloxacin (89.9%) but resistant against Tetracycline (62.2%) and Streptomycin (62.2%). *Staphylococcus* isolates showed 95.1 % Sensitivity for Levofloxacin and Gentamycin but 92.6 % of isolates were resistant against Oxacillin. Extended-spectrum beta-lactamase production was detected in 16.6% of gram-negative isolates, and Methicillin resistance were observed in 30% of *S. aureus* isolates.

Conclusion: The result demonstrated, the overall bacteriological quality of raw milk is poor which also contains pathogenic and antibiotic-drug resistant organisms; thus, the need for effective control measures to improve production, storage, and handling practices is implied.

Keywords: Raw milk, Dairy farms, Bacteriological quality, AMR.

1. Introduction

1.1 Background

Milk is an important source of nutrients for humans and animals. Milk is a yellowish white fluid of low viscosity, a slight sweet taste, and an opaque liquid, its secreted from mammary glands of female mammals[1,2].

Milk consist crucial nutrients , proteins , carbohydrates, lipids, vitamins, enzymes, dissolved gases, and dissolved salts(phosphates, nitrates, and chlorides of calcium), magnesium, potassium, and sodium [3] the constituents are use full for bodybuilding, body maintaining, bone health, and decreasing the risk of type II diabetes [2]. Furthermore milk contain also immunoglobulins, that protect the infants from several illness [4].

The nutrients, and high water activity of milk make it suitable growth and multiplication medium for many kinds of microorganisms that are pathogenic to consumers [5,6]. when suitable conditions exist, raw milk can be contaminated starting from the cows tit, pathogens shaded from diseased animals, and from the environment, [5,7].

Poor hygiene and sanitation in dairy farms, could introduce microorganisms to milk [8]. Also milk contamination is high for milk produced in traditional systems [9] ,because, in the traditional system, the overall practice in the food value chain is poor and there is no quality control measures but microbiological parameters can be used to assess how these processes have impacted milk quality and safety [10].

The safety of milk safety and production are intrinsically associated in the food value chain, from production to consumption [9]. Poor practices and chains of raw milk usage are very common in developing countries such as Ethiopia and this will be a threat to public health because consumptions of unsafe/raw milk are very high [11].

Raw milk usage and its impact is not well studied and documented, since currently raw milk consumption is becoming more common due to the insight of people that heating or pasteurization destroys the nutritional advantages of milk and bring some pre-determined outcomes such as lactose intolerance and allergic reactions [12,13].

In Ethiopia, raw milk consumption is a common practice particularly in rural areas, approximately about 4.4 billion liters of were produced in 2018 alone, out of this total production 70% (3.08 billion liters) was for human consumption; of this 57% was consumed by rural households and 43% were processed and distributed thorough different supply chains.

There are abundant, and different microbes in unpasteurized milk [14], such as *Salmonella* spp, *Shigella* ,*E. coli* o157:H7, and *Staphylococcus aureus*, are more repeatedly reported and identified as causes of food borne illness and outbreaks [15]. In different food stuffs including milk *E.coli* is particularly used as an indicator organism for fecal pollution and carry antimicrobial resistance genes [14,16].

The primary objective of this study is to investigate the bacteriological quality, prevalence pathogenic organisms with antimicrobial resistance profiles in raw milk samples obtained from selected dairy farms, this will fill the gap in knowledge and contribute to the development of evidence-based interventions to enhance milk bacteriological quality and mitigate the challenge's posed by antimicrobial resistance in the Ethiopia dairy sector which ensure safety of public health.

1.2 Statements of the problem

The health effect of contaminated unpasteurized milk consumption is determined by many factors, such as type of pathogenic bacteria, the amount of toxin(enterotoxin) produced , the immune status of the consumer such as immunocompromised persons, pregnant women's, elder persons, infants and children's [14,17].

Milk-borne infections can causes enteric illness such as diarrhea(bloody diarrhea) continuous vomiting fever and abdominal cramps, but small percentage of peoples will develop serious and chronic symptoms like hemolytic syndrome, reactive arthritis, scarlet fever sometimes even it become life threatening [18].

Currently many consumers concerned about food safety, markedly for diary and dairy products [19]. Raw milk and milk products also widely consumed here in Ethiopia [20], although they are not safe for consumer health since they provide suitable environment for the growth of germs. This is one of the reasons why, in addition to physicochemical testing microbiological testing and quality control also needed [21].

Food-borne illness affects up to 30% of the population in affluent countries each year, putting a burden on public health. In developed countries such as united states, there was 76 million incidents of food-borne disease are reported each year, resulting in 5,000 fatalities and 325,000 hospitalizations even though they have strong food chain supply system [22].

The pathogens in milk and food sources cause for losses in productivity and medical cost which is estimated around $35 billion each year. As the WHO report showed, diarrheal disease claimed the lives of 1.7 billion children under the age of five over the world, with a major portion of these deaths owing to contaminated food and water [23]. In southeast Asia and Africa, around 1.46 million (78%) deaths occurred from 1.8 million diarrheal cases [24].

In developing countries, diarrheal illnesses are a common public health challenge and play a significant role in infant malnutrition due to the fact that the decrease in absorption of essential nutrients during diarrheal illness [22].

Unpasteurized dairy and its products causes for many food borne outbreaks including in developed countries, in the United States, 202 outbreaks between 1998 and 2018 were linked to consumption of raw milk, compared, caused 2,645 illness and 228 hospitalization [25].

In Ethiopia, approximately 4.4 billion liters of fresh raw milk were produced but most of milk consumed is for household(57%) which is unregulated [20], but based on other studies consumption of unregulated milk and unpasteurized dairy products will have great the impact food-borne illness great [26].

Although there are some studies that determine the bacteriological quality of milk, still milk quality is assessed by physicochemical parameters, and there is also fewer data regarding the anti-bacterial resistance patterns of bacteria. As a result of improper utilization of antimicrobials in dairy animals, even normal microorganisms have become pathogenic, so this research have evaluated the bacteriological profile and drug resistance patterns of isolates in raw milk at selected dairy farms.

1.3 Significance of the study

The results obtained from this study will: -

- Generate information on bacteriological quality and antimicrobial resistance patterns of isolates from raw milk.
- Used to develop milk quality improvement programs for the dairy sector in Ethiopia.
- Provide additional data for further studies in raw milk bacteriological profile and antimicrobial resistance studies.
- Used for infection control and epidemiological purposes.
- help stakeholders and sectors formulate policy in the dairy industry.
- Provide information to the extension agents in the study area on the important practices to be taught farmers to ensure clean and high-quality milk production by small-scale dairy farmers.

2. Literature review

2.1 Milk composition and microbial growth

The consumption of unpasteurized milk in both developing and developed countries is higher; it's the first and sole nourishment for mammals [12]. Milk contain nutrients that are useful for humans and for growth of microorganisms [27].

Besides the nutritional content of raw milk, it is also composed of different mammary glands and cells, white blood cells, different active enzymes, and pathogenic and nonpathogenic microorganisms [28].Milk composition can be can be influenced by different aspects such as nutrition and dietary , genetics and environmental factors, level of milk production, lactation phase , disease (mastitis), cow's age, and season [29].

Raw milk will be prone to contamination after milking, when fresh raw milk reaches the collecting station or processing plant, the bacterial load (aerobic colony count) should be fewer than 50,000 per ml. To avoid excessive bacterial multiplication, milk should be produced, collected, handled, and transported hygienically [30].

The microbial contamination can be raised due to a diseased cow, unsanitary milking practices, poor personal hygiene, unsanitary contaminated utensils and/or milking equipment, and water supplied in sanitary activities, in addition pathogens from a cow's blood can enter the milk if it has an infectious stage/condition. The rate of new infections during milking is reduced when proper milking hygiene procedures are followed[31,32].

In raw milk and its derivatives, many pathogenic and indicator bacteria have possibilities to be present, that can occur at the time of collection, processing, distribution, and storage [33].

2.2 Microbial quality raw milk

Raw milk can have a chance to be contaminated at different stages of milk production, which is very common, especially in developing countries, this will pose a health risk and illness to the public different literatures demonstrated the contamination level of raw milk [34].

A cross-sectional study in the Republic of Russia has shown that a high microbial contamination level by pathogenic bacteria and coliform bacteria, A total of 60 samples were analyzed for microbial contamination. A significant number of raw milk samples (31.6%) exceed 10^6 cfu/ml. the most predominant bacterial microflora was Enterobacteriaceae. The highest total coliform count was found to be 10^7 cfu/ml, and 90% of samples had a count equal to 10^5 cfu/ml [35].

Another cross-sectional investigation aimed to assess raw milk from sales points in Poland has shown a higher occurrence of indicator bacteria, *E. coli* and *S. aureus* The study includes 50 samples from different sell points, to determine the microbiological characteristics of raw milk. The mean total aerobic bacterial counts were between 4.96–7.56 log cfu/ml and almost 98% of the sample exceeded the maximum tolerable limit for ACC(5 $\log_{10}$) Also, this study showed the Enterobacteriaceae count ranging from 1.81–6.23 $\log_{10}$ cfu/ml and *Klebsiella* spp, and *E.coli* were identified only in 12 samples out of 50 that were ranged from 5.0×10^1 to 1.1×10^2 cfu/ml [36].

A study by Samite Kakati in India also showed the contamination of raw milk by pathogenic microorganisms; among 200 samples, almost 50% of raw milk samples contained bacteria. The mean aerobic colony count ranges from1 4.62±0.25 to 10.59±0.07 $\log_{10}$ cfu/ml, and the total coliform count ranged from 7.50±0.07 to 7.32±0.10 $\log_{10}$ cfu/ml, which shows a high level of contamination [37].

Furthermore, cross-sectional study in small-holder dairy farms found in Tanzania, indicated that from 98 collected samples, above 50% were positive for microbial contamination. The mean aerobic counts were 11.02 ± 11.6 log cfu/ml, and that of coliform count was 6.7 ± 7.3 log cfu/ml, which was above the accepted limit of raw milk [38]. In this country between 2006 and 2014, another study shows the prevalence of *Salmonella*, *Staphylococcus* spp, *E.coli*, [39].

A study reported in Rwanda and Afghanistan demonstrated from total of 383 raw milk samples and bulk milk samples, the mean total bacterial count ranged from 1.1×10^6, and 1.6×10^7 cfu/ml, and in 14% milk samples *E. coli* was detected but *Salmonella* was not isolated in any of milk samples tested [40].

Similar study in Oromia, Ethiopia, found total aerobic bacterial counts of 6.88±0.46log cfu/ml, and the mean coliform counts value of 4.49 log cfu/ml, also in this study *E.coli* was founded to be 26(43.33%), and in 17(28.33%) of samples *S.aureus* was isolated but *Salmonella* spp was not isolated in any of raw milk sample [41].

According to a cross-sectional study in Diredawa, Ethiopia, the bacteriological analysis point out a mean total aerobic bacterial counts of 6.67 Log Cfu/ml and 1.24 Log cfu/ml coliform count from 60 raw milk samples from dairy farms [42].

Subsequent microbiological quality study in Bahirdar Ethiopia, discovered the overall mean coliform count was 7.11±0.13 log 10 cfu/ml, and *S. aureus* count 3.36±0.11 log 10 cfu/ml from a total of 80samples. The milk samples collected from rural areas, cooperatives highlighted poor microbiological quality and below bacteriological quality standards [43].

2.3 Antimicrobial resistance profile in raw milk

Antimicrobial resistance bacteria can be found in raw milk which can also resist pasteurization, these can be due to different factors such as from inappropriate practice on use of zoonotic antibiotics, less regulation and drug withdrawal period of cows [44].

Currently bacterial drug resistance is common which become serious public health concern due to the fact that, will leads to treatment failure, some bacteria's shows multi drug resistance patterns. Among Enterobacteriaceae family, *E. coli* is frequently found in milk and food stuffs, and transmit antibiotic resistance genes to other bacteria in raw milk [45].

A study in China showed higher degree of resistance in *E. coli* for different antimicrobials. *E.coli* isolates are resistant to penicillin (100%), sulphamethazole (53%), cephalosporin (32.5%) and ampicillin (30.1%)[45].

Similar study in Kenya showed an overall resistance of *E. coli* isolate for beta lactam drugs (81%), tetracycline (55%) and streptomycin (29 %), besides, the bacterial isolates show sensitive for Ciprofloxacin. 14.28% of the isolates showed resistance for three antibiotics and 14.2% have produced beta-lactamase enzyme [46].

Subsequent study in South Borena, Ethiopia demonstrated the resistant patterns of *E.coli* and *Salmonella* spp ,most of bacterial isolates (*E. coli* and *Salmonella* spp) were sensitive to fluoroquinolones and resistance were observed against ampicillin, in this study 14.3 % of isolates are resistant against three classes of antibiotics(i.e. multiple-drug resistance) [47]

Existing scientific evidence highlighted the poor bacteriological characteristics of raw milk in addition to the presence of antimicrobial drug resistance organism that can pose a serious illnesses and public health concern.

3. Objectives

3.1 General objective

- To evaluate bacteriological quality and anti-microbial resistance patterns of isolates from raw milk at selected dairy farms of Ethiopia.

3.2 Specific Objectives

- To determine bacteriological quality of raw milk using aerobic colony count, total coliform count, thermotolerant count, and *Staphylococcus* count.
- To determine the prevalence of *Salmonella, Shigella and S. aureus in* raw milk.
- To detect the anti-microbial resistance patterns of isolates from raw milk.
- To investigate the prevalence of extended spectrum beta lactamase production, methicillin resistance and multidrug resistance patterns of isolates from raw milk.

4. Materials and Methods

4.1 Study Area

The present study was conducted using raw milk samples that were collected from different dairy farms in Ethiopia. Ethiopia is a country located in the Horn of Africa, bounded to the north by Eritrea, to the west by Sudan, to the south by Kenya, and to the east by Somalia and Djibouti. It lies within the tropics between 3°24` and 14°53` North, and 32°42` and 48°12` East. It covers 1,120,000 square kilometers in 11 regional states, one city council, and one city administration [48].

The samples were collected from different dairy farms in Ethiopia that have a high potential for dairy cattle production and national milk shed sources, including Addis Ababa city, Oromia regions (Adama and Jimma), Amhara regions (Bahirdar and Gondar), Dire Dawa city administration, Sidama (Hawassa), and Wolaita-Sodo.

Ethiopia has the largest livestock population in Africa, including 55.02 million herds of cattle, in different livestock production systems with various agroecology (mixed, agro-pastoral, pastoral, semi-intensive, urban, and peri-urban) farming systems. Ethiopia is also the most populous country in Africa; the populations are on the rise with high expansion from rural areas to urban areas, which resulted in high demand for milk and milk products in urban areas [49].

4.2 Study period

The study was conducted from August 2022-April 2023 G.C.

4.3 Study Design

A Cross-sectional prospective study was conducted to assess the bacteriological quality, and anti-microbial resistance patterns of microorganisms in raw milk, and laboratory investigation was conducted to identify, characterize, and determine antimicrobial drug resistance of isolates.

4.4 Source population

All dairy farms that were found in national milk sheds of Ethiopia

4.5 Study population

Selected dairy farms that were found in national milk shades of Ethiopia.

4.6 Study variables

4.6.1 Dependent variables

Occurrence of bacteria in terms of aerobic colony count, total coliform count, thermotolerant coliform count, and *Staphylococcus* count, anti-microbial drug resistance patterns of bacteria.

4.6.2 Independent variables

Regions where the sample was collected, and the type of milk sample.

4.7 Inclusion and Exclusion criteria

4.7.1 Inclusion criteria

Raw milk samples from selected dairy farms during data collection period were included.

4.7.2 Exclusion criteria

Milk samples that are not collected from dairy farms, Milk samples collected from cows with known diseases, and cows that are on antibiotics, milk samples from improper containers for bulk tank milk. Milk sample with missing data, and label were excluded from the study.

4.8 Measurement and Data Collection

4.8.1 Sample size calculation

The sample size was determined by taking 11% occurrence of *S.aureus* in raw milk from Oromia region, Ethiopia [50].The confidence level at 95% and 5 % level of precision was taken into consideration. Then it was expressed using the expression below:

$$N= \left(Z\frac{\alpha}{2}\right) 2\ x\ P\ (1-P) \div d^2$$

Where:

N= the number of samples

Z = z-score (1.96)

P =incidence from previous study

d= Margin error; 0.05

Thus, it gives

$N= \underline{(1.96)^2 \text{ x } 0.11(1- 0.11)}$

(0.05) 2

≈ 151, by adding 10% contingency,

=167

Additional 9 bulk samples were collected from each dairy farms, a total of 176 raw milk samples were collected.

4.8.2 Sampling Method

Multi-stage sampling scheme were used, for selecting raw milk from samples that were collected for Zoonotic AMR Survey. The survey uses raw milk from different dairy, from Sidama region Southern nations, and national people's, Amhara, Oromia, Addis Ababa, and Diredawa city administrations. Dairy farms were selected by considering milk sheds, and high milk demanding populations.

Raw milk samples were selected using multi-stage sampling technique, firstly regions were included purposively based on national milk shades of Ethiopia. Then from each region 9 dairy farms were randomly selected. In this particular study, the total sample size (176) study samples) was distributed to four regions, and two city administrations based on their population proportion of cows (Table 1). Then fresh raw milk samples were selected from the survey sample that is collected directly from those selected farms at the same time.

Table 1 Sample size allocation to dairy farms based on dairy cow population proportion

S. №	Region	Dairy farm	Total cows in Dairy farms	Sample size
1.	Diredawa	Dairy Farm A	63	16
2.	Oromia	Dairy Farm B	140	33
3.	Amhara	Dairy Farm C	84	21
4.	Oromia	Dairy Farm D	45	12
5.	Oromia	Dairy Farm E	65	17
6.	SNNP	Dairy Farm F	42	13
7.	Sidama	Dairy Farm G	106	26
8.	Amhara	Dairy Farm H	50	14
9.	A. A	Dairy Farm I	98	24
		Total	693	176

4.8.3 Data Collection Procedure

4.8.3.1 Milk sample collection

About 100 ml raw milk sample was aseptically collected using sterile, screw capped bottle, the milk samples were collected from the cow's udder, and bulk tank, and placed in cold box. Then it was transported to Ethiopian Public Health Institute, Food microbiology laboratory for bacteriological analysis. All sample containers were labeled with sample Id , dairy farm ID, and type of milk sample [51].

4.8.4 Laboratory analysis

4.8.4.1 Aerobic colony count

About 25ml milk sample was suspended in 225ml Buffered peptone water (HiMedia Laboratories plc, Mumbai, India), and the milk homogenate (food homogenate) was serially diluted to five different dilutions using 9 ml of BPW for each dilution. From each dilution,1ml of raw milk homogenate was dispensed in sterile petrdish. Next 20ml of molten Standard Plate count agar (Hi Media Laboratories Pvt. Ltd, Mumbai, India) was poured into each petrdish. The plates were then incubated at 30c ^{0} for 72 hrs. All mesophilic bacteria in the plate were counted by plate counter, and the result was reported in cfu/ml by using dilution correction formula [52].

$$N = C \div V\ (n1 + n2)\ d$$

Where C = colony forming unit counted in each

V = volume of food homogenate

n_1= colonies counted first dilution

$n_{2=}$ colonies counted second dilution

d = dilution where the colonies counted

N= total plate count

4.8.4.2 Total coliform count

For the determination of coliform count 1 ml of food homogenate (enriched with BPW) from all dilution tube was transferred to sterile petrdish, and 10ml of molten Tryptone soya Agar (Hi Media Laboratories plc, Mumbai, India) was dispensed into perishes that contain the sample for enrichment of injured coliform bacteria. After an hour 20ml of molten Violet Red bile lactose agar (Hi

Media Laboratories plc, Mumbai, India) was overlayed onto TSA basement. The plates were incubated at 37^0c for 48hrs.Presumptive coliform count was recorded by enumerating each medium-sized and pinkish colony. To confirm the counted coliform, five typical colonies were inoculated to individual five Brilliant green bile lactose broth (Oxoid LTD, Basingstoke, Hampshire, England). The presence of gas production, and turbidity in the BGL broth tubes indicate presence of coliform bacteria. [53].

4.8.4.3 Thermotolerant coliform count

For the determination of Thermotolerant coliform count 1 ml of food homogenate (enriched with BPW) from all dilution tube was transferred to sterile petrdish, and 10ml of molten Tryptone soya Agar (Hi Media Laboratories plc, Mumbai, India) was dispensed into perishes that contain the sample for enrichment of injured coliform bacteria. After an hour 20ml of molten Violet Red bile lactose agar (Hi Media Laboratories plc, Mumbai, India) was overlayed onto TSA basement. The plates were incubated at 44.5^0c for 48hrs.Presumptive thermotolerant coliform count was recorded by enumerating each medium-sized and pinkish colony. To confirm the counted coliform, five typical colonies were inoculated to individual five *E. coli* broth (Oxoid LTD, Basingstoke, Hampshire, England). The presence of gas production, and turbidity in the EC broth tubes indicate presence of thermotolerant coliform bacteria[53].

4.8.4.4 *E. coli* test

For *E. coli* testing, from each confirmed thermotolerant coliform test that showed turbidity and gas production, a loopful of broth culture was transferred to another tube containing Nutrient broth (Oxoid ltd, Basingstoke, England). The tubes were incubated at 44.5^oc for 24 hrs. To confirm the presence of *E. coli* one drop of Kovacs reagent (HiMedia Laboratories Pvt. Ltd, Mumbai, India) was added to each tube. The tubes were examined for the development of red ring inside the tube indicate positive indole test and confirm the presence of *E.coli* [53].

4.8.4.5 *Salmonella*, and *Shigella* test

For *Salmonella* and *Shigella* testing,25 ml of raw milk was enriched using BPW (HiMedia Laboratories Pvt. Ltd, Mumbai, India), and the enrichment broth was incubated at 37^oc for 24hrs.After incubation, 1ml of diluted broth was transferred to Rappaport Vassiliadis broth (Oxoid LTD, Basingstoke, Hampshire, England) for selective enrichment at 42 ^{0}c for 24hrs.Following selective enrichment, a loopful of culture was streaked onto Xylose lysin deoxycholate agar (HiMedia Laboratories Pvt. Ltd, Mumbai, India) and incubated at 37^{o}c for 24hrs. Colonies that had typical colony characteristic i.e., pinkish colony with black head was confirmed by using VITEK-2 compact system (BioMérieux, France) [54].

4.8.4.6 *Staphylococcus* count

The count was done by spreading 0.1ml of milk homogenate on Mannitol salt agar plate (HiMedia Laboratories Pvt. Ltd, Mumbai, India), and the homogenate was distributed by spreading technique. After spreading, the MSA plates were incubated at 37^{0}c for 24 hrs., and colony with golden yellowish color was confirmed by tube coagulase test and further by VITEK-2 compact system [55,56].

4.8.4.7 Enterobacteriaceae testing

Enterobacteriaceae test was performed using ISO 21528 ,2017 method, 25ml of milk was suspended by 225 ml BPW, and incubated at 37^{0}c for 24 hrs. Then, one loop full culture was inoculated on VRBL agar using sterile loop, medium sized and pinkish colonies were further identified using VITEK 2 compact system [57].

4.8.4.8 Microbial identification using VITEK-2 compact

Microbial species identification was determined by VITEK-2 compact system (BioMérieux, France). This system contains automatic vacuum filler, sealing unit, incubator with reader (fluorescence detector), a computer control element and printer. The system detects bacterial growth by continuous monitoring of changes due to metabolism of bacteria in the each microwells of cards, GN ID and GP ID cards were used for the identifying bacterial isolates for Gram negative and gram positives respectively [58].

Bacterial suspension was prepared by emulsifying three pure colonies of bacteria in 0.45 saline, then the suspension was measured by densitometer to make the concentration of the suspension 0.5-0.63. The bacterial suspension along with identification cards were given to the machine and then system fill the cards automatically and the cards were transferred to another door for sealing and incubation. The VITEK-2 compact instrument kinetically monitor the change in microwell and interprets the results using its built in Advance expert system (AES) [59].

4.8.4.9 Antimicrobial sensitivity testing

Anti-microbial susceptibility testing was determined using Kirby-Bauer disk diffusion method. Bacterial suspensions were prepared by emulsifying three pure bacterial colonies from Nutrient agar (Oxoid LTD, Basingstoke, Hampshire, England), Subsequently, compared with 0.5 McFarland standard, and the colony was inoculated in all surfaces of Muller Hinton agar (Deben diagnostics, UK) using sterile swab., and incubated at 37^0c for 24 hours. Enterobacteriaceae isolates were tested against nine antibiotics(Mast-Group Diagnostics,UK) based on CLSI guideline, namely Amoxicillin clavulate (AMC:20/10µg), Ceftazidime (CAZ:30µg), Ciprofloxacin (CIP:5µg), Chloramphenicol (CHL:30µg), Meropenem (MER:10 µg), Streptomycin (STR:10 µg), Tetracycline (TR:30 µg), and Trimethoprim sulphathiazole (SXT:25 µg) and Gram positives were tested against Erythromycin (EM:10 µg), Clindamycin (CM:2 µg), Levofloxacin (LVX:5 µg), Chloramphenicol (CHL30 µg), Oxacillin (OX: 1 µg), Gentamycin (GM:10 µg), Tetracycline (TR:30 µg), Ampicillin(AMP:10 µg), and Trimethoprim Sulphamethazole (SXT:25 µg).zone of inhibition was measured to the nearest millimeter, and interpreted as Sensitive, intermediate and resistant based on Clinical & laboratory standard institute (CLSI 2022) guideline [60].

4.8.4.10 ESBL screening, and confirmation

Extended-spectrum beta lactamase(ESBL) production were analyzed according to CLSI guideline, for those Enterobacteriaceae isolate that showed reduced susceptibility or diameter of ≤ 22 millimeter for ceftazidime (30 µg) was considered as potential beta-lactamase producer, and confirmed by combination disc method (CDT), Ceftazidime(30ug) was placed with Ceftazidime with clavulanic acid (30 µg/10 µg) on an Muller Hinton Agar (MHA) plate inoculated with bacterial suspension of 0.5 McFarland standard, then incubated at 37^0c for 18 hrs. An increase in zone of inhibition zone diameter of >5 mm or 50% increment for a combination disc against ceftazidime disc alone was confirmed as extended spectrum beta-lactamase producer [61].

4.8.4.11 Methicillin Resistance *Staphylococcus aureus* (MRSA)

MRSA detection was conducted using CLSI guideline 2022, using Cefoxitin (30ug) disc. bacterial suspension (0.5Mcfraland standard) inoculated on MHA plate then Cefoxitin disc was placed. A diameter of ≤ 21 mm diameter was considered as methicillin-resistant (MRSA) [60,62].

4.9 Data Quality assurance

The quality of data assured in all quality assurance stage, standard operating procedures were prepared for data collection and laboratory detection. Raw milk samples were collected in a sterile container by aseptic technique. The bacteriological detection was carried out in a standardized laboratory. Bacteriological growth mediums were inoculated with known ATCC organisms (*E. coli* (ATCC 25922), and *S. aureus* (ATCC25923)). Good laboratory practice and universal safety guidelines were strictly followed. The results were recorded in result recording sheet before entered to Excel and checked for consistency and completeness.

4.10 Data analysis and interpretation

All the data was entered to Excel and transformed to log10, then the data analysis was performed using SPSS v 27 (Statistical product, and service solution). The data was checked for normality by Shapiro-wilk normality test. During analysis frequencies distribution and cross-tabulation was used. The data was presented using tables and different diagrams.

4.11 Ethical consideration

Ethical waiver was taken from the Department of Research, and Ethics Review Committee (DRERC) of Addis Ababa University College of Health Sciences, Department of Laboratory Sciences (protocol number, DRECR 694/22) and permission letter from Ethiopian public health institute was obtained before starting the thesis work. All information's obtained from the study were kept confidential.

4.12 Dissemination result

Th results of this study was submitted to Addis Ababa University, College of Health Sciences, and Department of Laboratory Sciences. The manuscript will also be submitted to peer-reviewed journals for publication.

4.13 Operational definition

Aerobic Colony Count (ACC): - total number of microbial loads that grow on standard plate count Agar under specific temperature and conditions.

Bacteriological quality of raw milk: the status of milk in terms of absence and presence of indicator organisms, and bacteriological profile of milk.

Bulk tank milk: - Raw milk sample that is collected from a container that contain all lactating cow's milk during collection time.

Thermotolerant coliforms: a group of Gram-negative bacteria that indicate contamination of bacteria from faecal origin, formerly they were called as fecal coliforms

Extended spectrum beta lactamase (ESBL) Enterobacteriaceae: It's a bacterium that produce enzymes against penicillinase (β-lactamase) and 1st,2nd, and 3rd generation cephalosporin.

Food microbiological quality: -refers to the quality of foods relative to product hygienic, and sanitary status, contamination of bacteria from soil, animal feces, that affect the safety of food.
Good quality: - when the values of ACC, Total coliform count, thermotolerant count and Staphylococcus count in acceptable limit.

Poor quality: -when the values of ACC above the recommended values $5*10^5$, presence of coliform, thermolortant coliform and *S. aureus* in food stuffs.

Individual cow's milk: -Raw milk sample that is collected from a single lactating cow.

Food microbiological Safety: -it refers to the presence of pathogenic organisms such as *Salmonella*, *shigella, S. aureus,* and drug resistant microorganisms in the food or in the product**.**

Microbiological safe milk: - absence of pathogenic organisms such as *Salmonella*, *shigella, S. aureus,* and drug resistant microorganisms in milk.

Microbiological unsafe milk: the presence of *Salmonella*, *shigella*, *S. aureus*, and drug resistant microorganisms in milk.

National Milk sheds: - the areas/dairy farms that supply most milk production for specific community.

Raw milk: -it's a type of milk that is not pasteurized, or it's the milk that obtained from direct udder of dairy animals**.**

Total coliforms: - a group of Gram-negative bacteria that indicate hygienic, and sanitary status, and the amount shows poor hygienic and sanitary condition.

Multi Drug resistance (MDR): - Drug resistance for at least one of the agents in three or more classes of antibiotic.

5. Result

5.1 Study sample description, and distribution

A total of 176 raw milk samples were collected from different dairy farms that are located in different regions of Ethiopia,35.4% of raw milk samples were collected from Oromia region, followed by Amhara region (28.7%), and SNNP region (22.5%).

Table 2: Distribution of raw milk samples from selected dairy farms in Ethiopia from August 2022 to April 2023.

S. №	Region	Number of dairy farms	Number of milk samples	Percentage
1.	Oromia region	3	61	35.4%
2.	Amhara region	2	51	28.7%
3.	SNNP region	2	40	22.5%
4.	Addis Ababa city administration	2	19	10.7%
5.	Diredawa City Administration	1	5	2.8%

Raw milk samples were collected from different dairy farms in National milk shades of Ethiopia. The sample was evenly distributed among 9 dairy farms in different regions (Table:2), from each dairy farm one bulk tank milk was collected for microbiological analysis, and the rest are collected from individual lactating cows (Fig 1).

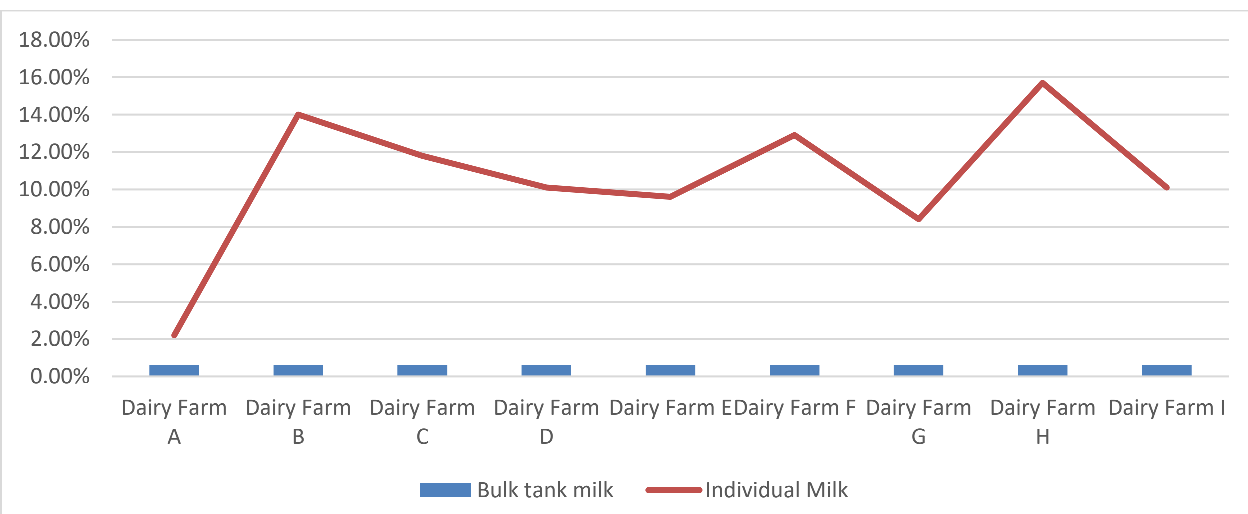

Figure 1: Distribution of raw milk sample in different region by milk sample type

5.2 Raw milk microbiological quality attributes

5.2.1 Aerobic colony count (ACC)

The mean ACC count was found to be 3.2±0.72 log_{10}cfu/ml, maximum value of 4.98 log_{10}cfu/ml were recorded from milk samples collected from dairy farm G (Table 3). Almost all samples have bacterial except for 7 milk samples.

Table 3: Mean ACC count (Log_{10} cfu/ml) of raw milk samples from selected dairy farms in Ethiopia from August 2022 to April 2023.

S. №	Dairy farm	Fre-quency	Mean ACC	SD	Lowest Value	Highest value
1.	Dairy Farm A	4	2.7099	0.22	2.51	3.00
2.	Dairy Farm B	26	3.0179	0.059	1.85	3.94
3.	Dairy Farm C	17	2.4606	0.62	1.60	3.90
4.	Dairy Farm D	18	3.2598	0.74	2.04	4.20
5.	Dairy Farm E	18	3.1745	0.57	2.20	4.04
6.	Dairy Farm F	22	3.1330	0.68	1.54	4.08
7.	Dairy Farm G	16	3.6085	0.52	2.90	4.75
8.	Dairy Farm H	29	3.4473	0.66	2.00	4.98
9.	Dairy Farm I	19	3.8337	0.67	2.40	4.70
10.	Total	169	3.2333	0.72	1.54	4.98

5.2.2 Total coliform count (TC)

In this study the TC of 176 milk samples was analyzed, but only 36 milk samples have confirmed total coliform count. The mean TC count was found to be 2.70 ±1.0 log_{10} cfu/ml, and the TC count ranged from 1.0 log_{10} cfu/ml to 4.48 log_{10}(Table 4); the maximum TCC value were 4.40 log_{10} cfu/ml which was collected from Diary farm I.

Table 4:Mean TC count (Log_{10} cfu/ml) of raw milk samples from selected dairy farms in Ethiopia from August 2022 to April 2023.

S. №	Dairy farm	Frequency	Mean TC	SD	Lowest value	Highest value
1.	Dairy Farm A	1	3.50	0	3.51	3.51
2.	Dairy Farm B	5	1.64	1.08	1.00	3.51
3.	Dairy Farm C	2	1.83	1.01	1.11	2.54
4.	Dairy Farm D	2	2.60	0.56	2.20	3.00
5.	Dairy Farm F	7	2.23	0.61	1.30	2.90
6.	Dairy Farm H	13	2.73	0.48	1.90	3.71
7.	Dairy Farm I	6	4.28	0.07	4.20	4.40
8.	Total	36	2.70	1.00	1.00	4.40

5.2.3 Thermotolerant coliform count (TCC)

In this study the TCC of 36 milk samples was analyzed which have total coliform count. but only 34 milk samples have confirmed thermotolerant coliform count. The mean TCC count was found to be 2.69 ±1.0 $\log_{10}$ cfu/ml the thermotolerant coliform count was ranged from 1.0 $\log_{10}$ cfu/ml, and 4.40 log10 cfu/ml.

Table 5: Mean TCC count (Log_{10} cfu/ml) of raw milk samples from selected dairy farms in Ethiopia from August 2022 to April 2023.

S. №	Dairy farm	Frequency	Mean TCC	SD	Lowest value	Highest value
1.	Dairy Farm A	1	3.50	.	3.51	3.51
2.	Dairy Farm B	4	1.12	0.25	1.00	1.51
3.	Dairy Farm C	1	2.30	.	2.30	2.30
4.	Dairy Farm D	2	2.60	0.56	2.20	3.00
5.	Dairy Farm F	7	2.09	0.70	1.30	2.90
6.	Dairy Farm H	13	2.73	0.48	1.90	3.71
7.	Dairy Farm I	6	4.28	0.08	4.20	4.40
8.	Total	34	2.69	1.02	1.00	4.40

5.2.4 *E. coli* type I count

The *E. coli* type I of 34 milk samples that thermotolerant coliform count was tested but only 22(39%) milk samples were confirmed *E. coli* type I count. The mean *E. coli* type I count were found to be 2.9±0.9 $\log_{10}$ cfu/ml with and ranged from 1.00 $\log_{10}$ cfu/ml to 4.40 log10 cfu/ml (Table 6).

Table 6: Mean *E. coli* type I counts ($\log_{10}$ cfu/ml) of raw milk samples from selected dairy farms in Ethiopia from August 2022 to April 2023.

S. №	Dairy farm	Fre-quency	Mean	SD	Lowest value	Highest value
1.	Dairy Farm A	1	3.50	.	3.51	3.51
2.	Dairy Farm B	1	1.0	.	1.00	1.00
3.	Dairy Farm D	2	2.6	0.56	2.20	3.00
4.	Dairy Farm F	4	2.1	0.84	1.30	2.90
5.	Dairy Farm H	9	2.8	0.50	1.90	3.71
6.	Dairy Farm I	5	4.3	0.07	4.20	4.40
7.	Total	22	2.9	0.99	1.00	4.40

5.2.5 *Staphylococcus* count

The presence *Staphylococcus* count was confirmed in 42(23.8%) of raw milk with mean count of 3.1 ±0.80.8 $\log_{10}$ cfu/ml and ranged from 1.51 $\log_{10}$ cfu/ml to 4.9 $\log_{10}$ cfu/ml (Table 7).

Table 7: Mean Staphylococcus count ($\log_{10}$ cfu/ml) of raw milk samples from selected dairy farms in Ethiopia from August 2022 to April 2023.

S. №	Dairy farm	Frequency	Mean	SD	Lowest value	Highest value
1.	Dairy Farm A	5	4.1	0.56	3.08	4.49
2.	Dairy Farm B	4	2.6	0.49	2.04	3.26
3.	Dairy Farm C	5	3.2	0.99	2.15	4.68
4.	Dairy Farm D	3	2.9	0.34	2.78	3.38
5.	Dairy Farm F	6	2.9	0.80	2.00	4.26
6.	Dairy Farm G	3	1.8	0.29	1.51	2.04
7.	Dairy Farm H	6	3.7	0.78	2.43	4.90
8.	Dairy Farm I	10	3.0	0.69	2.26	4.56

9.	Total	42	3.1	0.85	1.51	4.90

5.3 Raw milk safety microbiological attributes

5.3.1 *Salmonella*, and *Shigella*

In this study, *Salmonella* species was isolated only in one milk sample from dairy farm A. but *Shigella* spp was not isolated per 25 ml of milk sample in both bulk, and individual milk sample.

5.3.2 *S. aureus*

From 176 raw milk sample tested, 24(13.6%) were positive for *S. aureus*, and 18(10.2%) were coagulase negative *Staphylococcus* group of bacteria (Fig 2) using VITEK-GP card (BioMérieux, France)

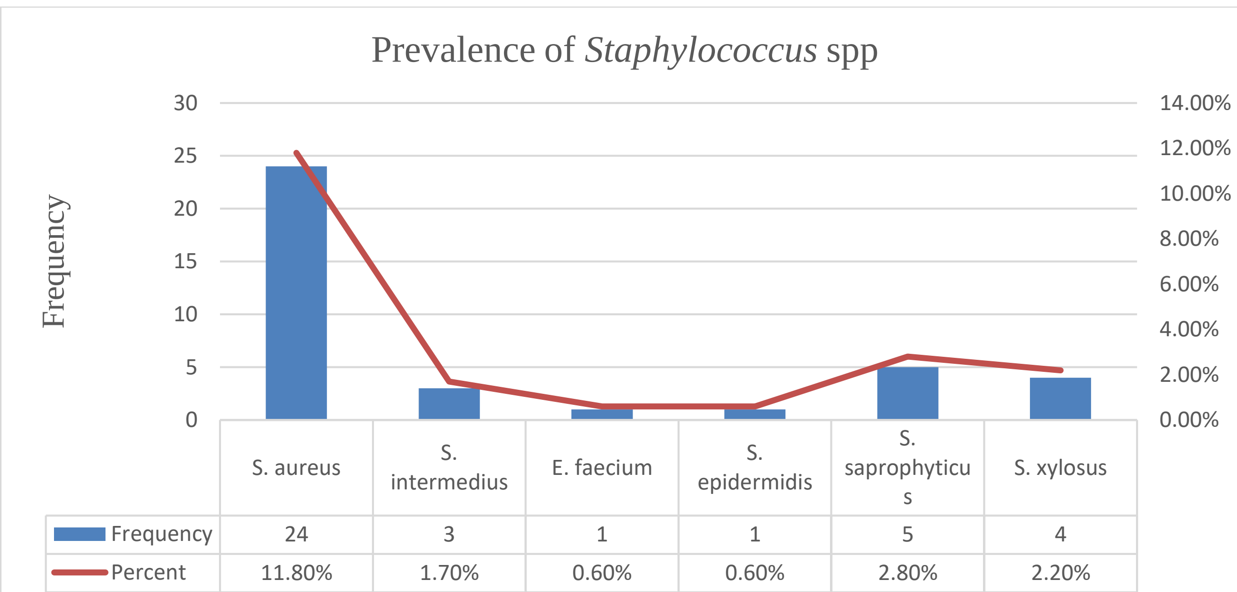

Figure 2 Prevalence of Staphylococcus spp in raw milk samples at selected dairy farms in Ethiopia from August 2022 to April 2023

5.3.3 Enterobacteriaceae

Among 69 different Enterobacteriaceae isolated from raw milk samples, the prevalence of *E. coli* (77%) and 5% *Klebsiella* spp and *E. Cloacae* each.

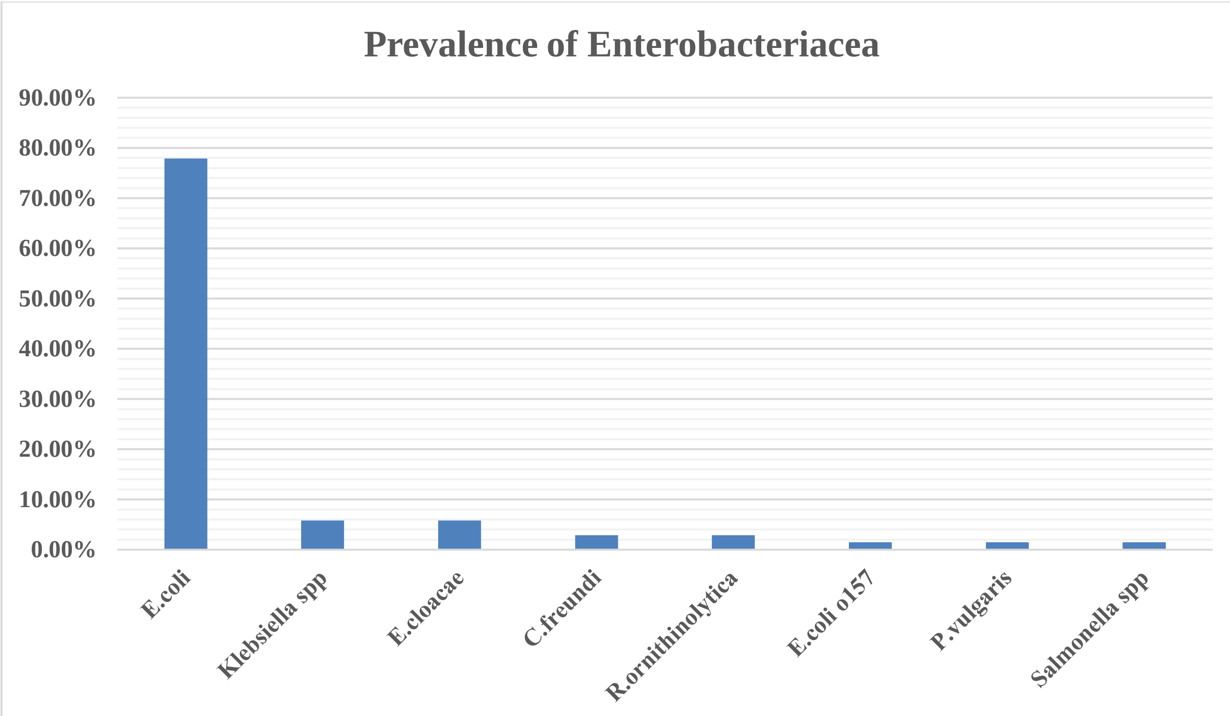

Figure 3: Prevalence Enterobacteriaceae in raw milk samples from selected dairy farms in Ethiopia from August 2022 to April 2023.

5.4 Antimicrobial resistance patterns bacteria isolated from raw milk

5.4.1 Antimicrobial resistance patterns of Enterobacteriaceae

A total of 69 Enterobacteriaceae isolates were tested against 9 antibiotics based on CLSI guideline, namely Amoxicillin clavulate (20/10µg), Ceftazidime (30µg), Ciprofloxacin (5µg), Chloramphenicol (30µg), Meropenem (10 µg), Streptomycin (10 µg), Tetracycline (30 µg), and Trimethoprim sulphathiazole (25 µg) by considering intrinsic resistance of organisms.

The result showed that the most effective(sensitive) antibiotic tested was Meropenem (96.8%) followed by ciprofloxacin (89.9%), and Chloramphenicol (89.9%). Besides the sensitivity patterns of bacteria, resistance was observed in Tetracycline (62.3%) followed by Streptomycin (62.2%).

Table 8: Antimicrobial sensitivity patterns of Enterobacteriaceae from raw milk at selected dairy farms in Ethiopia from August 2022 to April 2023.

Antibiotic list	Sensitivity status	*E. coli(n=55)*	*C. Freundii (n=2)*	*E. coli o157 (n=1)*	*E. cloacae(n=4)*	*K. pneumoniae(n=2)*	*K. oxytoca (n=2)*	*P. vulgaris (n=1)*	*Salmonella species (n=1)*
AMC	S	33(60%)	1(1.4%)	0	4(5.8%)	2(2.9%)	2(2.9%)	0%	1(1.4%)
	I	16(23.2%)	1(1.4%)	0%	0%	0%	0%	0%	0%
	R	6(8.7%)	0%	1(1.4%)	0%	0%	0%	1(1.4%)	0%
CAZ	S	40(63.7%)	2(3.2%)	1(1.6%)	4(6.3%)	1(1.6%)	2(3.2%)	1(1.6%)	1(1.6%)
	I	9(14.3%)	0%	0%	0%	0%	0%	0%	0%
	R	0%	0%	0%	0%	1(1.4%)	0%	0%	0%
CIP	S	49(71%)	1(1.4%)	1(1.4%)	4(5.8%)	2(2.9%)	2(2.9%)	1(1.4%)	1(1.4%)
	I	5(7.2%)	1(1.4%)	0%	0%	0%	0%	0%	0%
	R	1(1.4%)	0%	0%	0%	0%	0%	0%	0%
CHL	S	50% (72.5%)	2(2.9%)	0%	4(5.8%)	2(2.9%)	2(2.9%)	0%	1(1.4%)
	I	1(1.4%)	0%	0%	0%	0%	0%	1(1.4%)	0%
	R	4(5.8%)	0%	1(1.4%)	0%	0%	0%	0%	0%
MER	S	54(78.3%)	2(2.9%)	1(1.4%)	4(5.8%)	2(2.9%)	2(2.9%)	1(1.4%)	1(1.4%)
	I	1(1.4)	0%	0%	0%	0%	0%	0%	0%
STR	S	18(26.1%)	1(1.4%)	0%	2(2.9%)	1(1.4%)	1(1.4%)	0%	1(1.4%)
	I	2(2.9%)	0%	0%	0%	0%	0%	0%	0%
	R	35(50%.7%)	1(1.4%)	1(1.4%)	2(2.9%)	1(1.4%)	1(1.4%)	1(1.4%)	0%
TR	S	18(26.1%)	1(1.4%)	0%	2(2.9%)	1(1.4%)	1(1.4%)	0%	1(1.4%)
	I	2(2.9%)	0%	0%	0%	0%	0%	0%	0%
	R	35(50%.7%)	1(1.4%)	1(1.4%)	2(2.9%)	1(1.4%)	1(1.4%)	1(1.4%)	0%
SXT	S	47(68.1%)	2(2.9%)	0%	3(4.3%)	2(2.9%)	2(2.9%)	0%	1(1.4%)
	I	3(4.3%)	0%	0%	0%	0%	0%	0%	0%
	R	5(7.2%)	0%	1(1.4%)	1(1.4%)	0%	0%	1(1.4%)	0%

1=Sensitive, 2=Intermediate,3=Resistant AMC=Amoxicillin clavulate (20/10µg), CAZ=Ceftazidime (30µg), CIP=Ciprofloxacin (5µg), CHL=Chloramphenicol (30µg), MER=meropenem (10 µg), STR=streptomycin (10 µg), TR=tetracycline (30 µg), and SXT=Trimethoprim sulphathiazole (25µg)

5.4.1.1 ESBL and Carbapenem's producing isolates

Extended Beta Lactamase (ESBL) test was performed for 60(56 *E. coli* and four *Klebsiella*) isolates, by combination disc (CDT) method. In this study 19(31.6%) isolates were screened as potential ESBL producers and the confirmation test shows only 10(16.6%) isolates are ESBL producers.

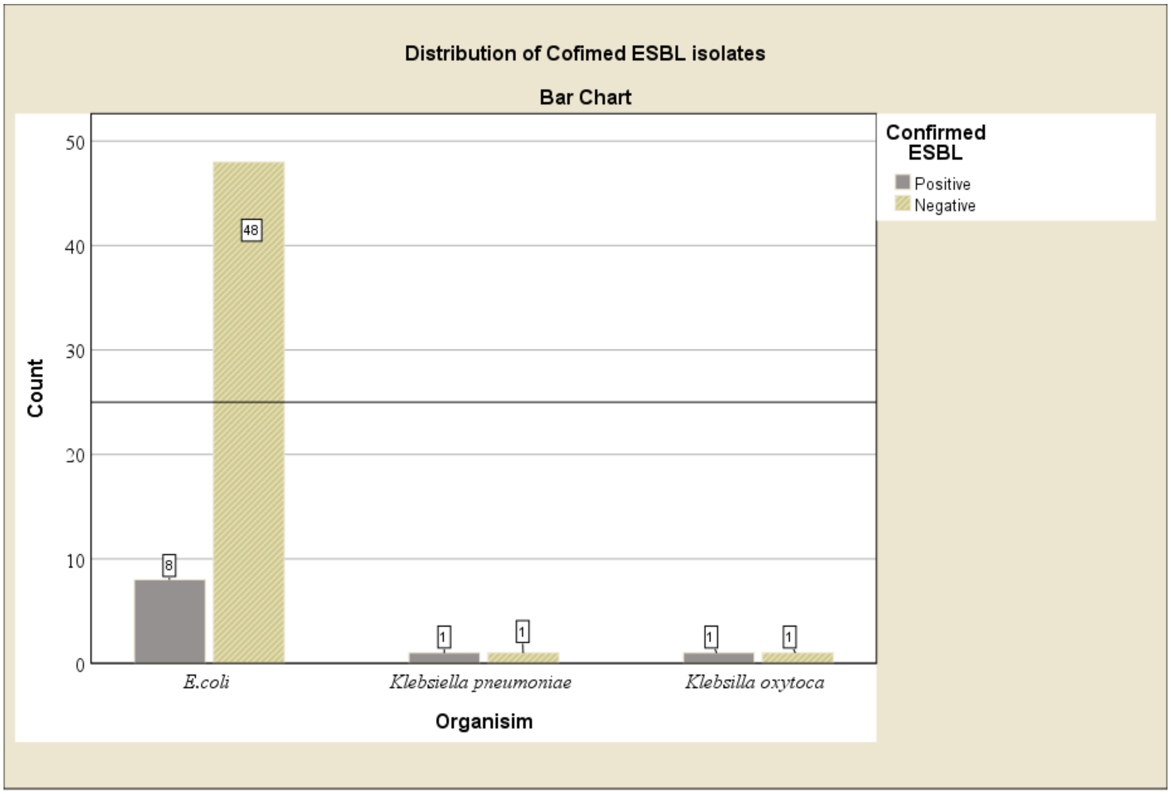

Figure 4:ESBL results of isolates from raw milk samples at selected dairy farms in Ethiopia from August 2022 to April 2023.

In this study none of the isolates exhibit resistance for Meropenem(10µg), but one isolate was intermediate.

5.4.1.1.1 Prevalence of MDR Enterobacteriaceae

The outcomes of antibiotic sensitivity test, further characterized based on their resistance patterns, 43(62.4%) resistance were observed was observed for two antibiotics and but MDR was observed in 11 (16%) of isolates.

Table 9: Distribution of multi drug resistant Enterobacteriaceae isolated from raw milk samples at selected dairy farms in Ethiopia from August 2022 to April 2023.

S. Nº	Organism	R_3	R_4	R_5	MDR status
1.	*E. coli* (55)	3(4.3%)	3(4.3%)	1(1.4%)	R5
2.	*P. vulgaris*(n=1)	0	1(1.4%)	0	R4
3.	*E. cloacae*(n=4)	1(1.4%)	0	0	R3
4.	*E. coli* o157(1)	0	0	1(1.4%)	R5
Total		4(5.80%)	5(7.20%)	2(2.9%)	

R_0 = Sensitive R_3=Resistance for three antibiotics, R_4=Resistance for four antibiotics, R_5= Resistance for five

The isolates showed different resistance pattern but resistance for Tetracycline (30 µg), Streptomycin (10 µg), and Chloramphenicol (30µg) were observed frequently (Figure:3).

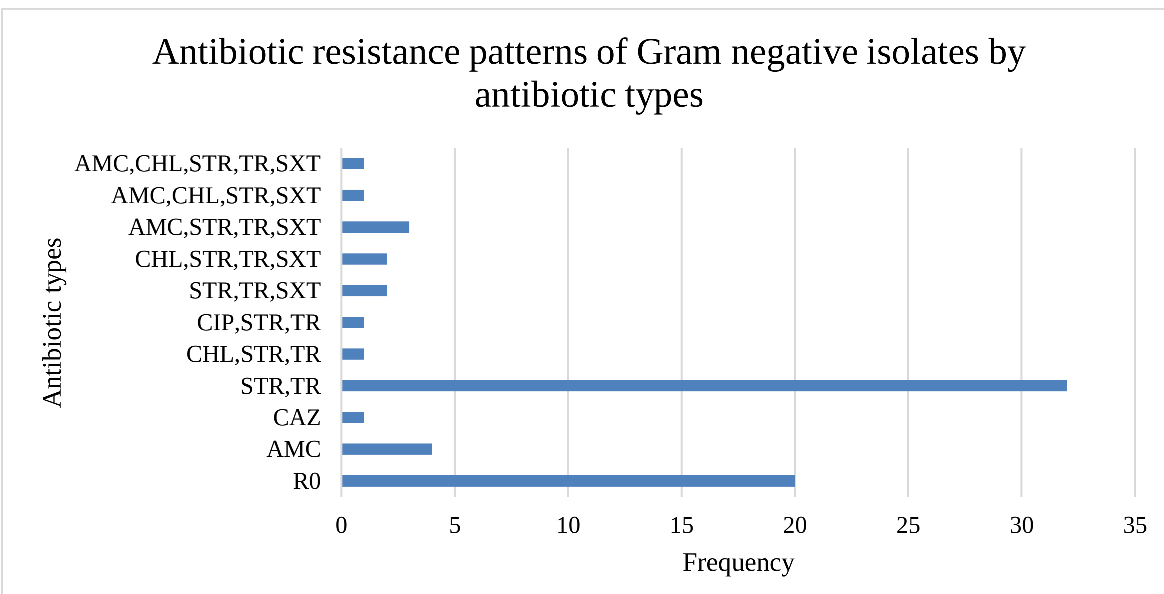

Figure 5:Distribution of MDR Enterobacteriaceae by drug types isolated from raw milk samples at selected dairy farms in Ethiopia from August 2022 to April 2023.

5.4.2 Antimicrobial resistance patterns of *Staphylococcus* bacteria

A total of 42 Staphylococci isolates were tested against, Erythromycin (10 µg), Clindamycin (2 µg), Levofloxacin (5 µg), Chloramphenicol (30 µg), Oxacillin (1 µg), Gentamycin (10 µg), Tetracycline (30 µg), Trimethoprim Sulphamethazole (25 µg) and Cefoxitin (FOX:30µg) was used for Methicillin resistance (MRSA) testing.

Table 11: Antimicrobial sensitivity patterns of Staphylococcus spp from raw milk at selected dairy farms in Ethiopia from August 2022 to April 2023.

Anti-biotics	Sensitivity status	*S. aureus* (n=20)	*S. intermedius* (n=3)	*E. faecium* (n=1)	*S. saprophyticus*(n=4)
ERM	S	18(43.9%)	3(7.3%)	0	4(9.8%)
	I	1(2.4%)	0	0	0
	R	1(2.4%)	0	1(2.4%)	0
CM	S	15(36.6%)	3(7.3%)	1(2.4%)	4(9.8%)
	I	1(2.4%)	0	0	0
	R	4(9.8%)	0	0	0
LVX	S	19	3(7.3%)	1(2.4%)	4(9.8%)
	I	0	0	0	0
	R	1(2.4%)	0	0	0
CHL	S	19(46.3%)	2(4.9%)	1(2.4%)	3(7.3%)
	I	1(2.4%)	0	0	0
	R	0	1(2.4%)	0	1(2.4%)
GM	S	19(46.3%)	3(7.3%)	0	4(9.8%)
	R	1(2.4%)	0	1(2.4%)	0
OX	S	3(7.3%)	0	0	0
	R	17(41.5%)	3(7.3%)	1(2.4%)	4(9.8%)
TR	S	9(22%)	1(2.4%)	1(2.4%)	1(2.4%)
	I	3(7.3%)	1(2.4%)	0	0
	R	8(19.5%)	1(2.4%)	0	3(7.3%)
SXT	S	18(43.9%)	2(4.9%)	0	4(9.8%)
	I	1(2.4%)	0	0	0
	R	1(2.4%)	1(2.4%)	1(2.4%)	0
AMP	S	1(2.4%)	0	0	2(4.9%)
	R	19(46.3%)	3(7.3%)	1(2.4%)	2(4.9%)

S=Sensitive, I=Intermediate= Resistant

EM=Erythromycin (10 µg), CM=Clindamycin (2 µg), LVX=Levofloxacin (5 µg), CHL=Chloramphenicol (30 µg), OX= Oxacillin (1 µg), GM=Gentamycin (10 µg), TR=Tetracycline (30 µg), AMP=Ampicillin (10 µg), SXT=Trimethoprim Sulphamethazole (25 µg).

The result showed that 95.1% of isolates were sensitive to levofloxacin and Gentamycin, followed by 92.6% for clindamycin and Chloramphenicol. On the other side 92.6% of isolates were resistant to Oxacillin and Tetracycline (39%).

Antibiotic susceptibility results of *S. aureus* indicated,95% of isolate's were sensitive to Levofloxacin and Chloramphenicol, followed by Oxacillin (85%) and Tetracycline (40%).

5.4.2.1 Prevalence of MRSA

Methicillin resistant *staphylococcus aureus* test (MRSA) was conducted using Cefoxitin (30µg) drug, from a total 20 of tested *S. aureus* isolates 6(30%) of them was resistant for Cefoxitin (30µg), which is interpreted as MRSA. In addition, 18(51.1%) of Coagulase negative *Staphylococcus* isolates exhibit Oxacillin/methicillin resistance.

5.4.2.2 Prevalence of MDR *Staphylococcus* isolates

In this study *Staphylococcus* bacteria were further characterized based on their resistance patterns, the prevalence of MDR was found to be 22(53.6%) from tested isolates, 39 % of resistance pattern were observed for three antibiotics (Oxacillin (1 µg), Tetracycline (30 µg), and Ampicillin (10 µg)).

Table 10 Distribution of Multi drug resistant *Staphylococcus* bacteria isolated from raw milk at selected dairy farms in Ethiopia from August 2022 to April 2023.

S. No	Organism	R_3	R_4	R_5	Total
1.	Aerococcus Virdans	0	1(2.4%)	0	3(7.3%)
2.	Enerococcus faecium	0	0	1(2.4%)	1(2.4%)
3.	Staphylococcus aureus	10(24.4%)	1(2.4%)	0	20(48.8%)
4.	Staphylococcus intermedius	1(2.4%)	1(2.4%)	0	3(7.3%)
5.	Staphylococcus lentus	2(4.9%)	1(2.4%)	0	3(7.3%)
6.	Staphylococcus saprophyticus	2(4.9%)	0	0	4(9.8%)
7.	Staphylococcus xylosus	0	1(2.4%)	0	3(7.3%)

R_3= resistant against three classes of antibiotics, R_4=resistant against four classes of antibiotics, R_5= resistant against five classes antibiotics

6. Discussion

Infection caused by pathogenic, antibiotic drug resistant and MDR bacteria have a great threat for public health especially for children, pregnant women, lactating women , and also it could have significant impact on veterinary industry , and country growth[63].

The results of aerobic colony count indicated the overall bacterial load in milk sample, [64]. In this study the mean ACC count was 3.2 ±0.72 log_{10} cfu/ml. The Ethiopian standard agency recommends an ACC below 200,000 cfu/ml [65].Remarkably majority of the raw milk samples(97%) were below the limit, indicating satisfactory quality or hygiene of milk. However, four milk samples exceeded the acceptable limit which suggest poor quality and inadequate hygiene practices. A study in India showed a similar ACC count with mean value of 3.2 log 10 cfu/ml count[66]. But a study in Addis Ababa, Ethiopia showed a higher mean ACC count of 6.5log_{10}cfu/ml [67], and another study in Nekemete town showed a higher ACC count which was 7.42 log_{10} cfu/ml [68].

The Total coliform count indicate presence of indicator bacteria from organic matter , and environment in milk sample, in the current study the mean TC count was 2.70 log_{10} cfu/ml, but according to the ESA, the recommended TC limit was <3 log_{10} cfu/ml or 1000 cfu/ml but majority of tested samples were <3 log10 cfu/ml , the results of total coliform count, indicate poor quality of raw milk, this findings are slightly lower than a study conducted in Eastern wollega Ethiopia (3.14 log_{10} cfu/ml) [69], in Algeria (4.6 log_{10} cfu/ml) [21], and in Namibia (3.4 log_{10} cfu/ml0 [70]

The results of thermo-tolerant coliform, and *E. coli* type I count indicate direct contamination of raw milk by human feces, the mean E. *coli* type I count was determined to be 2.9 log_{10} cfu/ml, World health organization doesn't allow occurrence of *E. coli* in raw milk, but 22(13%) raw milk samples were above the recommended limit.

The presence of *E. coli* type I implies poor quality of raw milk and it may cause diarrhea, continuous vomiting in infants, and children. The finding of this study agrees with a study conducted in North west Gonder, Ethiopia with the mean value of 3.20 log_{10} cfu/ml, but the study doesn't evaluate the antimicrobial sensitivity patterns of *E.coli* isolates[71]. In contrast, a higher mean *E.coli* count (3.93 log_{10} cfu/ml) was reported in Sudan [72].

Bacteriological safety testing or prevalence of *Salmonella*, *Shigella* and *S. aureus*, were determined, and the result indicated lower incidence of *Salmonella* and *Shigella*, only one raw milk sample contain *Salmonella* spp. The growth of this bacteria's can be affected by many growth inhibitors, the current study revealed that there is a low incidence of *Salmonella*, and *Shigella* in raw milk sample.

Unlike the findings of our study, a higher incidence of *Salmonella* spp were shows up in Wolaita, Ethiopia, that revealed 9.3% occurrence from 151 raw milk samples from dairy farms [73],another study in SNNPR, Ethiopia also reported 8.3% occurrence of *Salmonella* species from 384 raw milk samples [74].

Lower incidence of *Salmonella* spp also reported in Algeria, *Salmonella* spp were not isolated from 144 raw milk samples [21]. Similar study in Turkey also reported low prevalence of *Salmonella*, although they use Maldi-toff machine for isolation of foodborne pathogens [75].

The occurrence of Enterobacteriaceae in raw milk will indicate potential health hazard due to consumption of raw milk [76].The result showed that a higher incidence of *E.coli* (77%). other bacteria's such as *Klebsiella spp* (5%) and *E. cloacae* (5%) were also isolated.

In contrast with our findings, study conducted in Egypt shown the most frequent members of isolated were *H.alive* (30.95%), *S. liquefaciens* (25.0%), and *K.pneumonia* (15.48%) [76]. Another study in Pakistan also showed the occurrence of *Klebsiella* (20%), *Salmonella* (23%), *Proteus* (18%), *E. coli* (17%), *Enterobacte*r (13%) *Shigella* (4%) , and *Yersinia* (4%) from 81 raw milk samples [77].

The results of *Staphylococcus* count indicate contamination of raw milk from environment and food handlers during production. In Ethiopia most of studies don't determine *Staphylococcus* count and also its not categorized as milk quality indicator [39,47,78].

The current study revealed the mean *Staphylococcus* count was determined to be 3.1 log_{10} cfu/ml , and the highest value were 4.9 log_{10} cfu/ml but European Commission recommends below 2000 cfu/ml[79] but majority of the raw milk samples were above the microbiological acceptable limit, Similar to our finding a higher mean (4.24 log_{10}cfu/ml) *Staphylococcus* count was reported in Holeta town, Oromia region, Ethiopia [50] and consistent with this, higher mean count also reported in Tigray, Ethiopia with mean count of 4.31log_{10} cfu/ml [80].

The overall occurrence of *Staphylococcus* bacteria was found to be 42(23.8%), among these 24(13.6%) isolates were *S. aureus* and 18(10.2%) were coagulase negative *Staphylococcus*. In food sample the amount of *S.aureus* count above >4 log_{10}cfu per ml will have a probability for production of entero-toxin which bring a major food safety concern, in this study 10(6%) of raw milk samples have >4log_{10} cfu/ml *S.aureus* count[81].

A study in Holeta, Oromia region, Ethiopia revealed the prevalence of *S.aureus* to be 33(8.64%) from 383 raw milk samples, this study showed relatively lower prevalence than that of our study , and 15(48.4%) out of 31 tested samples have count greater than 4 log_{10} cfu/ml [50].A study in Tigray, Ethiopia showed a higher occurrence (47%) from 163 raw milk samples [80].

Antimicrobial susceptibility test for Enterobacteriaceae isolates showed a higher resistance against Tetracycline(30 µg) and Streptomycin(10 µg) which accounts 62.3 % .In contrast with this, a study in Tanzania, reported 95.6% resistance against Amoxycillin, 31.1% resistance against Amoxycillin/clavulanic acid(20/10 µg), 24.4% resistance against Tetracycline(30 µg), and 2.2% resistance against Ciprofloxacin(5 µg) [31].

In our study almost all isolates (96.8%) were sensitive for Meropenem(10 µg) ,followed by 89.9% for Ciprofloxacin (5 µg), and Chloramphenicol(30 µg),which is in line with a study conducted in Tanzania[31], and also a study in Borana Oromia region showed 100% sensitivity of *E.coli* isolates for Gentamicin(10 µg), Ciprofloxacin(5 µg), and Chloramphenicol(30 µg) [47]. A study in Jigjiga, Ethiopia showed antimicrobial resistance of *E. coli* isolates,42.3 % against Doxycycline (30 µg), 30 % against Ampicillin (10 µg), and Gentamycin (10 µg) [82].

Almost all Gram-negative isolates were sensitive for Meropenem (10 µg), but only one isolate was found to be intermediate. Similar findings were reported in Bangladesh regarding Carbapenem resistance with 100% sensitivity for Imipenem(10 µg) but 100% resistance against Tetracycline(30 µg) [83].

In current study MDR were observed in 43(62.3%) of bacterial isolates against three different classes of antibiotics, most of them were resistant against Tetracycline (30 µg) , and Streptomycin(10 µg)) which accounts46.4%,followed by resistance for four antibiotics (Amoxicillin clavulate (20/10µg) ,Streptomycin(10 µg), Chloramphenicol(30µg) ,and Sulphamethazole trimethoprim(25µg)) which accounts 4.3%.but a study in Tanzania demonstrated 91.2% MDR isolates from raw milk [31].

ESBL production was observed in 16.6% isolates, this finding is consistent with a study conducted in India,12(54.4%) *E.coli* isolates exhibit ESBL [84]. In contrast, a study in Sudan demonstrated a higher occurrence of ESBL, from a total of 58 isolates,43(74.1%) were found to be ESBL producers [85].

The Antimicrobial sensitivity patterns of *Staphylococcus* bacteria showed 95.1% sensitivity for levofloxacin, and Gentamycin (95.1%) followed by 92.6% for Clindamycin (2 µg), and Chloramphenicol (30 µg). Among the tested isolates, 92.6% resistance was observed against Oxacillin (1 µg) ,73.2% against Ampicillin (10 µg), and 39% against Tetracycline(30 µg) .Similar finding was reported in Oromia, Ethiopia, in which 95% resistance against antibiotics in Penicillin class and 80% against Cefotaxime(30 µg) [50].

Another study in Hawassa showed, higher resistance (70.9%) against Ampicillin (10 µg) 60.3% against Oxacillin (1 µg) and 30.9 % against Amoxicillin-Clavulanic acid (20/10 µg). and 100% sensitive to Ciprofloxacin (5 µg) [74].similar finding was observed in a study in Bangladesh which accounts 100% resistance for Rifampicin, but 100% sensitivity to Clindamycin(2 µg), Chloramphenicol(30 µg), and Ciprofloxacin(5 µg)[83].

Multiple drug resistance *Staphylococcus* isolates was detected in 35(85.4%) isolates in three different classes of antibiotics, the dominant resistance patterns of the isolates were resistance for three antibiotics namely oxacillin (1 µg), Tetracycline (30 µg) , and Ampicillin (10 µg) which accounts 29 % , and resistance pattern for Two antibiotics (Oxacillin (1µg), and Ampicillin (10 µg)) was 19.5 %.But a study in Oromia, Ethiopia revealed 62.5% MDR *Staphylococcus* isolates , with dominant resistance pattern for four antibiotics namely Ampicillin(10 μg), Oxacillin(1 μg), Cefotaxime(30 μg), and Tetracycline(30 μg) [50]

In this study, from a total 20 tested *S. aureus* isolates 30% of them exhibit resistance for Cefoxitin(30 μg) which is interpreted as Methicillin resistant *S. aureus* (MRSA), but a study in Uganda showed higher prevalence(72.7%) occurrence of MRSA [86],and 60% occurrence were also reported in Hawassa, Ethiopia [87].

The result indicated a high aerobic plate count, and other attributes which shows poor microbial quality of milk, and a significant resistance pattern of bacteria showed for commonly utilized drugs.

7. Strength and limitation of the study

7.1 Strength of the study

- Identification of bacteria was done by automated VITEK-2 compact system which enable accurate species identification.
- A standard technique was used for microbiological tests, and antibiotic sensitivity testing.
- Phenotypic antimicrobial patterns were conducted for isolates from Raw milk

7.2 Limitations of the study

- Antibiotic sensitivity testing was conducted only phenotypically using Disc diffusion technique
- The result of VITEK-2 compacts, for *Salmonella*, and *E. coli* o157:H7 were not confirmed with anti-sera due to shortage of resources.
- The study doesn't assess environmental factors, and other factors due to shortage of resources.

8. Conclusion and Recommendation

8.1 Conclusions

The current study indicated that the overall quality of raw milk was poor. The presence of pathogenic organisms in raw milk highlights a potential health risk to consumers, furthermore this study revealed antimicrobial resistance among bacterial isolates particularly for Tetracycline, Oxacillin and Amoxicillin Clavulate.

The detection of Extended spectrum beta lactamase production, Methicillin Resistant Staphylococcus aureus (MRSA), and Multi-drug resistance strains in raw milk indicate the urgent need for appropriate measures on antibiotic usage.

In conclusion the results of this study highlighted suboptimal quality of raw milk from dairy farms, the emergence of antimicrobial resistance and pathogen transmissions. Thus, its crucial to create awareness for consumers.

8.2 Recommendations

Regulatory bodies and Stakeholders should

- Jointly work together to support dairy farmers in their pursuit of high-quality milk production through legislative enforcement, quality-based incentives, and extension programs;
- Enact national milk quality standards, and regulations that define the minimal operational conditions, routine husbandry methods, and health criteria for dairy animals.
- Create awareness for consumers consumption of raw milk.
- Well, designed, and coordinated control programs for minimizing the risk of milk borne pathogens, and antimicrobial resistance.
- Enforce to meet regulatory milk hygienic standards
- Be a regular visit by extension workers/veterinarians into small scale dairy farm to create awareness, and give training on hygienic production of milk.

Research institutes, Researchers, and Non-Governmental organizations should

- Work jointly to establish surveillance system in the country, and molecular epidemiological studies.
- Work Further research on resistance genes, and virulence gene to assess the circulating gene in the community.
- Strengthen Food safety Laboratories in terms of materials, laboratory automated equipment that aid identification, and AMR detection.
- Collaborate with different institute, and departments to study environmental factors in one health platform.

Farmers, Food handlers, and dairy processing workers should

- Manage the farm, processing plant sanitation, and hygiene practices when producing, and storing raw milk

- Follow good manufacturing practice to decrease potential source of contaminations.
- Use antiseptic, disinfectant, and clean all milk utensils before, and after using.
- Clean hands, cow’s udder, towels, and milk utensils

Consumers, and general public should

- Use pasteurized products rather than using raw milk because pasteurization doesn’t affect the nutritional status of the milk
- Effective storage, and handling practices to prevent contamination.
- Use heat treatment that is equivalent to pasteurization technique.

9. Reference

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Annexes 1: Raw milk sample collection form

S. No	Date, and Time of collection	Type of sample (Individual or Bulk)	Sample ID	Cows ID	Region	Collected by
1.						
2.						
3.						
4.						
5.						
6.						
7.						
8.						
9.						

Annexes 2: Raw milk sample microbiological result registration form

S. No	Date re-ceived	Sam-ple ID	Lab code	Types of sam-ples	APC	PCC	CCC	PFC	CFC	E. coli	*Salmo-nella*	Shi-gella	S. au-reus	Re-mark

Annexes 3: Method of sample collection, labelling, storing, and dispatching

The first step of lab operation is collection, transportation, handling, and storage of milk samples following a standard process in order to maintain the quality of milk samples that is fit for testing purpose. In the following sections, a standard process has been explained for the lab, and field staff.

3.1 General requirement for sample collection

Make arrangement of all necessary materials before going to sample collection. Please take the following items for collection of milk samples.

- Clean, dry, leak-proof, sterile container (mainly plastic) with graduation/calibration on the body, and polythene zip bag;
- Glass Beakers, 100 ml
- A cool box/ thermos flask to carry the sample;
- Personal protective clothing like apron, gloves, mask, etc.;
- Sticker tags, marker, note pad, mask, sanitizers, and biohazard bag;
- A disposal bag for carrying disposable materials like leftover milk, gloves, mask, etc.;
- A h, and sanitizer to sanitize h, ands of the sample collector;

3.2 Information to be collected along with the sample

The following information need to be collected from the household/source at the time of sample collection:

- Type of the sample (e.g., milk/curd /cream/others);
- If bulk milk is collected: Time of milking;
- Weight/ volume of the sample
- Place of collection
- Date, and time of collection;
- Name, and designation of the collector;
- Purpose of collecting the samples;
- All samples should be marked with a unique sample number

The above information shall be recorded against the specific sample number allocated to each sample collected, and part of the information shall be supplied with the sample to the lab.

General considerations in sample collection, handling, and storage

the samples should never be touched with bare hands.

- Gloves, and mask should always be used in the process of collection. •
- Sample should not be exposed to dirty materials/environment after collection, and should not be mixed with other biological samples.
- Disinfect the surface of the work area before opening the samples for measuring, packaging, etc. at the laboratory
- Sample should preferably be measured directly in the sterile container with graduation;
- Gloves, mask, and other materials in contact with the sample must be disposed properly.
- The stopper/cover of the container shall be securely fastened to prevent leakage of the contents in transit.

Method of milk sample collection from milk container

In order to collect milk sample for testing purpose, following methods should be followed

- Agitate the liquid milk thoroughly before sample is taken in order to make the contents of a milk container as homogenous as possible for obtaining a representative sample.
- Never agitate too vigorously because air bubbles, if dispersed in milk, will change its physical properties, and disturb the analysis.
- In order to make sure that a sample will well represent the whole contents of milk can take the half of the required sample from the lower portion, and another half from upper part of the milk can.
- To take sample from a smaller milk container, turn the container upside down few times before sampling ensuring the container is closed well.
- Agitate the sample carefully again before the sample start to analysis in a laboratory.

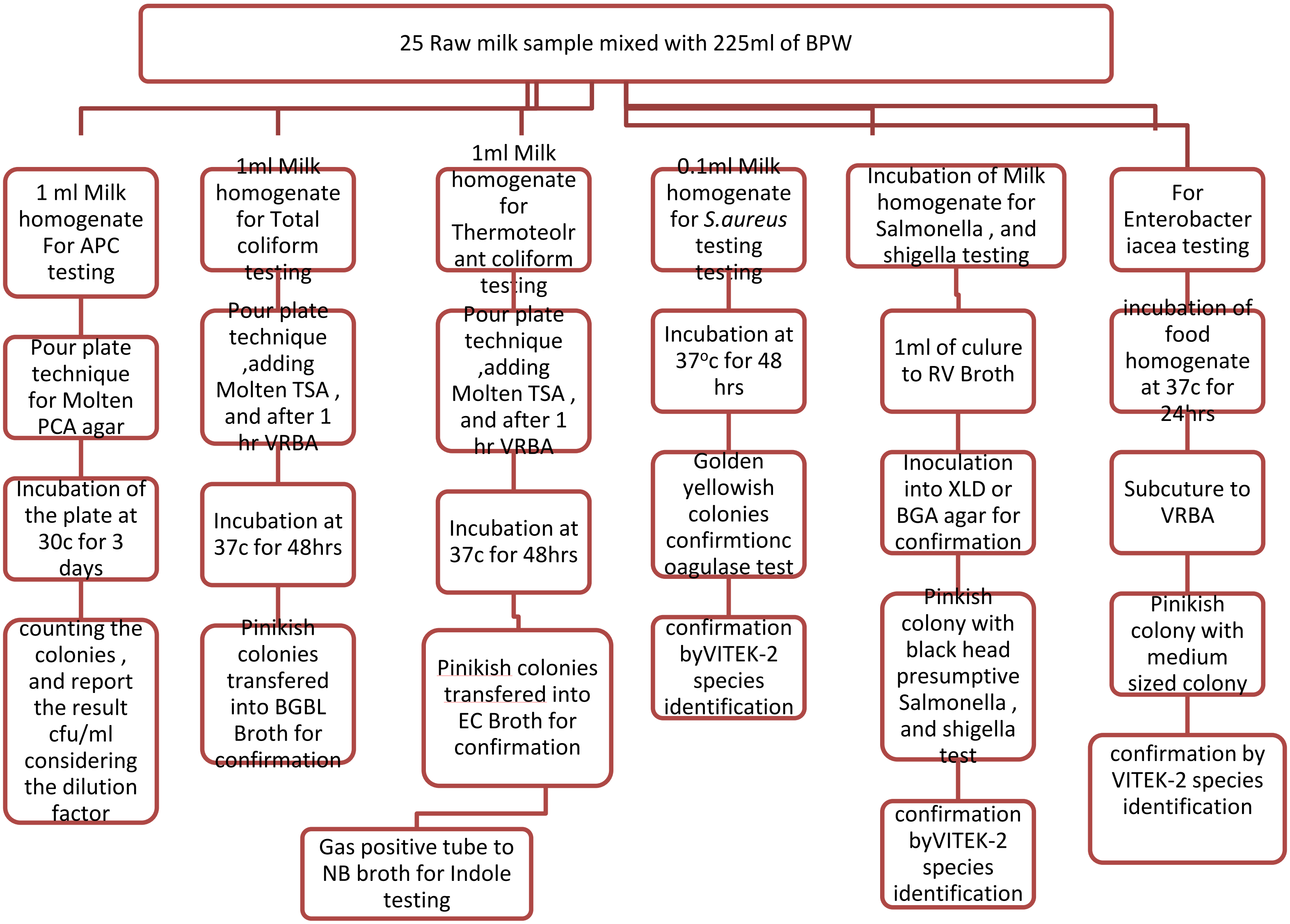
Collecting milk sample directly from cow

To collect milk sample directly from a cow, the following procedure shall be followed-

- Ask the owner of the cow to clean the udder, and teats of the cow thoroughly with water.
- Put on the clean gloves, face mask, apron, etc. Strip two to three streams of milk from each teat in order to flush the teat canal, and thereby to reduce contamination risk.

- Dry teats thoroughly with an individual cloth towel, paying close attention particularly to the teat end
- Open the milk vial, and immediately take the sample, making sure not to touch the inside of the tube or bottom part of the lid. Hold the milk vial about 3 inches from the teat end, and fill the tube half to three-quarters full of milk. Hold the vial at a 45-degree angle to prevent dirt from falling into the vial.
- Put the sample in cool box immediately.

Annexes 4: Microbial detection flow diagram



Annexes 5: Declaration

I, the undersigned, declare that this M.Sc. thesis is my original work, has not been presented for a degree in this or any other university, and that all sources of materials used for the thesis have been duly acknowledged.

M.Sc. Candidate: Kaleab Sebsibe (B.Sc.)

Signature: ___________________

Date of submission: ___________________

This thesis has been submitted with our approval as advisors.

Advisor: Kassu Desta (MSc, PhD candidate, Associate professor)

Signature: ____________________

Date: ____________________

Place: Addis Ababa, Ethiopia.

Advisor: Dessie Abera (MSc, PhD Candidate)

Signature: _____________________

Date: _____________________

Place: Addis Ababa, Ethiopia.