Infection and Drug Resistance

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ORIGINAL RESEARCH

Pathogenic Bacteria and Their Antibiotic Resistance Patterns in Milk, Yoghurt and Milk Contact Surfaces in Debre Berhan Town, Ethiopia

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Background: Bacterial contamination of milk is a primary culprit for causing foodborne illnesses, presenting a significant health hazard for millions of individuals around the globe. The level and variety of microorganisms present in raw milk determine its degree of contamination and the potential health risks it poses.

Methods: A cross-sectional survey was conducted from February to August. A questionnaire was used to collect data on socio-demographic characteristics and hygiene practices from milk distributors and traders. Raw milk, yoghurt, swabs from milk containers and drinking cups were collected and processed for bacterial isolation and identification, antibiotic susceptibility testing, MDR screening and confirmation, ESBL screening and confirmation. Finally, all data were pooled and analyzed using SPSS software version 25.

Results: A total of 120 samples of fresh milk, yogurt and cotton swabs from milk containers and cups were collected. A total of 80 bacterial isolates were isolated from 120 samples. Among the bacteria isolated, *S. aureus* 17 (21.3%), *E. coli* 17 (21.3%), *S. epidermidis* 14 (17.5%), *Klebsiella* spp. 9 (11.3%) and *Salmonella* spp. 7 (8.8%) were detected most often. High rate of contamination was observed in fresh milk 23 (28.8%) and yogurt 23 (28.8%). All isolates were resistant to at least one antibiotic tested. Comparatively, high rates of resistance were observed in all isolates to the most commonly prescribed antibiotics in Ethiopia. However, lower rates of resistance have been observed for recently introduced antibiotics in Ethiopia. Of the isolates, 20 (25.0%) were resistant to eight or more antibiotics. While 16 (20.0%), 12 (15.0%), 9 (11.3%) isolates were resistant to two, three and five antibiotics, respectively. Of the bacteria isolated, 52/80 (65.0%) were MDR, 25/49 (51.0%) were screened for ESBL production, and 20/49 (40.8%) isolates were confirmed as ESBL producer.

Conclusion: This study showed a high rate of bacterial isolates along with MDR and ESBL-producing strains in raw milk, yoghurt, milk container swabs and drinking cup swab samples, associated with poor hygiene and sanitation practices.

Keywords: bacterial contamination, multidrug-resistance, extended-spectrum beta-lactamase, raw milk, yoghurt, milk contact surface

Introduction

The human burden of food-borne disease is still poorly understood.[1] Over the past decade, most countries have seen a significant increase in the incidence of food-borne disease.[2] Dairy products such as milk and yogurt, which are common foods in many countries, provide a favourable environment for the growth of many microorganisms due to their nutritional content.[3] Many studies have been conducted to improve raw milk quality, reduce the risk of microbial contamination, and improve the chemical and nutritional quality of dairy products.[3–5] Today, daily consumption of milk and dairy products is becoming increasingly popular due to potential benefits such as rich nutrients, beneficial bacteria, and prevention of lactose intolerance are increasingly welcomed. However, due to the possible presence of pathogens and their toxins, consumption of raw milk can pose a significant risk of food-borne disease.[6–9]

Received: 18 May 2023
Accepted: 27 June 2023
Published: 3 July 2023

The dairy products that are produced from raw milk are often found to contain *Staphylococcus aureus, Salmonella* spp., *Listeria monocytogenes*, and *E. coli*, which are among the most common pathogens.[4,8] In addition, *Staphylococcus aureus, Listeria monocytogenes*, and *Salmonella* spp. may contribute to bovine mastitis and can be directly excreted in milk.[8–11] According to the World Health Organization (WHO), foodborne pathogens were responsible for approximately 600,652,361 cases and 418,608 deaths worldwide in 2010.[12] The highest burden of foodborne illness per capita has been reported in Africa, with a median foodborne disability-adjusted life-year (DALY) of 2455 per 100,000 populations.[13] Among these cases, 26.6% were attributed to *Salmonella* spp., 11.2% to *Enteropathogenic E. coli*, 8.6% to *Enterotoxigenic E. coli*, 0.08% to *Listeria monocytogenes*, 5.7% to *Campylobacter spp*., and 0.004% to Shiga-toxin-producing *E. coli*.[12,13]

In developing countries, especially Ethiopia, milk is a major cause of food-borne disease. This happens when milk and various dairy products are produced under unsanitary conditions and poor production practices.[14] Although contamination control of raw milk and dairy products is not routinely practiced, the Ethiopian dairy industry is evolving towards a market-based system.[15] A survey conducted in central Ethiopia found that 31.8% of the farmers consumed raw milk.[16] In the dairy market value chain, unsanitarily processed milk is easily contaminated by milk-borne bacterial pathogens, making it a convenient carrier for disease transmission and posing a significant public health risk to consumers.[17–19] A study conducted in northern Ethiopia found that milk contamination rates ranged from 45% to 75%.[20]

Overdose, misuse and long-term use of pharmaceuticals to treat animals and humans have led to alarming growth and prevalence of antibiotic-resistant bacteria. This exacerbates the clinical situation and poses one of the greatest medical challenges of our time, contributing to poor cure rates, loss of human and animal life, and animal dairy products.[21] Therefore, this study was designed to assess bacterial milk contamination, and the resistance patterns of bacterial isolated from raw milk, yoghurt, and contact surfaces in Debre Berhan Town, Ethiopia.

Methods

Study Design and Area

The cross-sectional survey was conducted from February to August 2022 in the town of Debre Berhan, 130 km northeast of Addis Ababa. In the town, consumers brought most of their milk and dairy products directly from farmers, traders/traders and cafeterias. People in the town of Debre Berhan and the surrounding villages also regularly consume milk and dairy products.

Data and Sample Collection

A questionnaire was used to collect data on socio-demographic characteristics and hygiene practices from vendors and milk handlers. Data on facility sanitary conditions were collected through individual interviews and observations. Four types of samples were collected: fresh milk, yoghurt, milk container cotton swabs, and drink cup cotton swabs. An equal number of samples (30 each, 120 total) were randomly collected from farmers, vendors, or cafeterias. Fifty millilitres of raw milk and yogurt were collected. Environmental swab samples from milk containers and drinking cups were collected over an area of 30 cm^2 by using cotton swabs soaked with sterile buffered peptone water (BPW).

Sample Processing, Isolation and Identification of Bacteria

Approximately 1 mL of raw milk and yogurt samples were transferred to sterile test tubes containing 9 mL of BPW. Cotton swab samples from the milk container and drinking cup were placed in a sterile test tube and suspended in a test tube containing 9 mL of BPW. All samples were labelled, placed in sterile plastic bags and transported to Debre Berhan University Microbiology Laboratory. The mixture was then serially diluted. Finally, 0.1 mL volumes of diluted samples were aseptically taken and inoculated on solidified MacConkey (Oxoid Ltd., Basingstoke and Hampshire, UK) and mannitol salt agar (Oxoid Ltd.) using the pour plate method. After pure colonies were obtained and key characteristics were recorded, the isolated organisms were further identified using a series of biochemical tests. Gram-negative bacteria were identified based on colonial morphology and pigmentation, oxidase test, carbohydrate fermentation, H_2S production, citrate utilization, motility, growth at 42°C, indole formation, lysine decarboxylase and lysine deaminase

production, and urea hydrolysis. Gram-positive isolates were also differentiated by colonial characteristics, catalase test coagulase tests, and novobiocin susceptibility test.

Antimicrobial Susceptibility Testing

Antimicrobial resistance profiles of the isolates were determined using the standard Kirby–Bauer disk diffusion method described by CLSI-2022.[21] Bacterial cultures were prepared by suspending freshly cultured bacteria in 4–5 mL of sterile saline and adjusting the turbidity to the McFarland standard turbidity of 0.5. After standardizing the bacterial suspension, a sterile cotton swab was soaked and twisted several times with firm pressure against the inner wall of the tube to remove excess liquid. The dry surface of Mueller Hinton agar plates (Oxoid Ltd.) was inoculated by spreading a cotton swab across the surface. The antibiotic disc was then placed onto the inoculation plate using sterile forceps and incubated overnight (18–24 hours) at 37°C. Bacterial isolates were tested for the antibiotics commonly prescribed in Ethiopia, in accordance with the Ethiopian Ministry of Health Antimicrobial Prescribing Policy. The antibiotics tested were amoxicillin (AMC, 30µg), ampicillin (AMP, 10µg), penicillin (P, 10µg), cotrimoxazole (SXT, 30µg), ciprofloxacin (CIP, 5µg), chloramphenicol (CAF, 30µg), gentamicin (CN, 10µg), erythromycin (E, 15µg), tetracycline (TC, 30µg), doxycycline (DXT, 30µg), methicillin (MET, 5 µg), ceftriaxone (CRO, 30µg), imipenem (IMI, 10µg), meropenem (MRP, 10µg), cefotaxime (CTX, 30µg), and ceftazidime (CAZ, 30µg).

Multidrug-Resistant Isolates

Bacterial strains resistant to one or more antibiotics from three or more antibiotic classes are considered multidrug resistant.[22]

Confirmation of ESBLs-Producing Bacteria

Enterobacteriaceae isolates with reduced susceptibility and resistance to cefotaxime and/or ceftazidime were included as potential ESBL producers. Isolates with a ceftazidime (30 µg) zone of inhibition size of ≤22 mm and/or a cefotaxime (30 µg) zone of inhibition size of ≤27 mm were considered potential ESBL producers.[21] To confirm ESBL production, ceftazidime (30 µg) and cefotaxime (30 µg) discs alone and in combination with clavulanic acid (30 µg/10 µg) were placed 25 mm centre to centre on Mueller–Hinton agar overlaid with the bacterial suspension and incubated overnight (18–24 hours) at 37 °C. Bacterial isolates were identified as ESBL producers that increased the zone of inhibition diameter of the combined discs by more than 5 mm compared to ceftazidime or cefotaxime discs alone.[21]

Quality Control

Prior to the actual work, reagents were checked for proper functioning and handled according to standard procedures. *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus (*ATCC 25923) were used as quality control organisms throughout the antimicrobial susceptibility testing. For ESBLs confirmatory test, ESBLs positive *K. pneumoniae* ATCC 700603 and ESBLs negative *E. coli* ATCC 25922 control strains were used.

Statistical Analysis

Data obtained from questionnaires and laboratory procedures were summarized and analyzed using SPSS software version 25. Cross tabulation was performed, and quantitative values (frequency and percentage) are shown in the statistical table.

Result

Socio-Demographic and Hygienic Practice of the Study Participant

All milk and swabs of milk contact surfaces were collected from farmers (5, 16.7%), vendors (8, 26.7%) and cafeterias (17, 56.7%). All persons working on milk business (100%) did not have any formal training on milk handling and marketing. Majority of the business owners 22 (73.3%) had a habit of mixing milk from different farms or sources. Most of the business owners 15 (50.0%) waited less than 1 hour to received their milk, but milk for sale was held back for 1–2

hours by 11 (36.7%) of the milk business owner. Similarly, 17 (56.7%) of the business owners took around a day to complete the milk. Most of the correspondents clean the milk containers and utensil daily 23 (76.7%) with hot water with detergent/soap 16 (53.3%) (Table 1).

Table 1 Socio-Demographic, Hygienic Practice of Vendors and Cafeteria at Debre Berhan Town, Ethiopia, 2022

Variables	Category	Frequency	Percent
Type of business	Farmer	5	16.7
	Vendors	8	26.7
	Cafeteria	17	56.7
Any kind formal training on milk handling and marketing	Yes	0	0.0
	No	30	100.0
Habit of mixing milk from different farms or sources	Yes	22	73.3
	No	8	26.7
Time usually take to transport the milk from sources	≤1hours	15	50.0
	1–2 hours	7	23.3
	≥	8	26.7
Time usually take to keep milk from transport until sale	≤ 1hours	9	30.0
	1–2 hours	11	36.7
	≥ 2hours	10	33.3
Equipment used to store the milk	Glass container	2	6.7
	Aluminum container	8	26.7
	Plastic container	9	30.0
	Refrigerator	11	36.7
Time usually take to finish the milk	1 day	17	56.7
	2 days	11	36.7
	>2 days	2	6.7
Frequency of cleansing the milk containers and utensil	Daily	23	76.7
	Weekly	0	0.0
	Infrequently	7	23.3
Means of cleansing the milk container and utensil	Cold water only	3	10.0
	Hot water only	4	13.3
	Cold water with detergent/soap	7	23.3
	Hot water with detergent/soap	16	53.3

(*Continued*)

Table 1 (Continued).

Variables	Category	Frequency	Percent
Hand washing	Cold water only	14	46.7
	Hot water only	0	0.0
	Cold water with detergent/soap	16	53.3
	Hot water with detergent/soap	0	0.0
Source of water for washing	Tap water	30	100.0
	Other	0	0.0

Bacterial Contamination of Milk, Yoghurt and Milk Contact Surfaces

A total of 80 bacteria were isolated from 120 samples. Of the isolates, 31 (38.8%) were Gram-positive and 49 (61.2%) were Gram-negative. Among the nine different bacterial species isolated, *S. aureus* 17 (21.3%), *E. coli* 17 (21.3%), *S. epidermis* 14 (17.5%), *Klebsiella* spp. 9 (11.3%) and *Salmonella* spp. 7 (8.8%) were most frequently detected (Table 2).

The rate of contamination was high in raw milk (23, 28.8%) and yoghurt (23, 28.8%). Among gram-positive bacteria species, *S. aureus* was the predominant isolate in raw milk (4, 23.5%), yoghurt (5, 29.4%), and milk container swabs (5, 29.4%). *S. epidermidis* was also the predominant isolate from milk container swabs (5, 35.7%) and drinking cup swabs (7, 50.0%). *E. coli* was found to be the most common enteric bacterium in raw milk (8, 57.1%). The same is true for *Salmonella* spp. (4, 57.1%). Other pathogens, such as *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Proteus* spp., *and Shigella* spp. were also isolated from milk, yoghurt and milk contact surfaces (Table 3).

Antibiotic Resistance Patterns of Isolates in Milk, Yoghurt and Milk Contact Surfaces

The highest level of resistance was observed in ampicillin (79, 98.8%), amoxicillin (75, 93.8%), and penicillin (30, 96.8%). For example, all isolates except *S. epidermidis* (13, 92.9%) were 100% resistant for ampicillin. Also, the most isolated bacterial species like *S. epidermidis* (14, 100.0%), *Salmonella* spp. (7, 100.0%), *Citrobacter* spp. (5, 100.0%), *Shigella* spp. (5, 100.0%), *S. aureus* (16, 94.1%), *E. coli* (16, 94.1%) *and Klebsiella* spp. (8, 88.9%) were the most

Table 2 Prevalence of Bacterial Contamination in Milk, Yoghurt and Milk Contact Surfaces at Debre Berhan Town, Ethiopia, 2022

Isolates	Prevalence	Percent
S. aureus	17	21.3
S. epidermidis	14	17.5
E. coli	17	21.3
Klebsiella spp.	9	11.3
Enterobacter spp.	5	6.3
Salmonella spp.	7	8.8
Citrobacter spp.	5	6.3
Proteus spp.	5	6.3
Shigella spp.	1	1.3
Total	80	100.0

Table 3 Distribution of Bacterial Contamination in Milk, Yoghurt and Milk Contact Surfaces at Debre Berhan Town, Ethiopia, 2022.

Isolates	Raw Milk	Yoghurt	Milk Container Swab	Drinking Cup Swab
S. aureus (17)	4(23.5)	5(29.4)	5(29.4)	3(17.6)
S. epidermidis (14)	0(0.0)	2(14.3)	5(35.7)	7(50.0)
E. coli (17)	8(57.1)	4(23.5)	3(17.6)	2(11.8)
Klebsiella spp. (9)	2(22.2)	3(33.3)	2(22.2)	2(22.2)
Enterobacter spp. (5)	2(40.0)	3(60.0)	0(0.0)	0(0.0)
Salmonella spp. (7)	4(57.1)	2(28.6)	1(14.3)	0(0.0)
Citrobacter spp. (5)	2(40.0)	2(40.0)	0(0.0)	1(20.0)
Proteus spp. (5)	1(20.0)	2(40.0)	1(20.0)	1(20.0)
Shigella spp. (1)	0(0.0)	0(0.0)	1(20.0)	0(0.0)
Total (80)	23(28.8)	23(28.8)	18(22.5)	16(20.0)

Note: Data are represented as N(%).

resistant pathogen against amoxicillin. Gram-positive bacteria also showed the highest level of resistance for penicillin (30, 96.8%) (Table 4).

Generally, high rate of resistance was observed in all isolates for the most prescribed antibiotics in Ethiopia. For example, (53, 66.3%), for erythromycin, (46, 57.5%), for cotrimoxazole, (47, 58.8%) for doxycycline, (42, 52.5%) for ceftriaxone, (40, 50.0%) for gentamycin and (45, 56.3%) for chloramphenicol a high rate of resistance was observed. However, lower resistance rate was observed for lately introduced antibiotics to Ethiopia like meropenem (16, 20.0%), imipenem (19, 23.8%), ceftazidime (24, 30.0%) and ceftaxime (27, 33.8%) (Table 4).

Among the isolates, 20 (25.0%) were resistant for eight and more antibiotics (eg, *S. aureus* (5, 29.4%), *E. coli* (5, 29.4%), *S. epidermidis* (4, 23.5%), and *Enterobacter* spp. (2, 40.0%)), while 16 (20.0%), 12 (15.0%), and 9 (11.3%) of the isolates were resistant for two, three, and five, antibiotics, respectively (Table 5).

Multiple Drug-Resistant and ESBL-Producing Bacterial Isolates in Milk, Yoghurt and Milk Contact Surfaces

Among isolated bacteria, 52/80 (65.0%) were MDR, 25/49 (51.0%) were screened for ESBL production and 20/49 (40.8%) isolates were confirmed as ESBL producer. *Citrobacter* spp. *was* 100% MDR followed by *Enterobacter* spp (4, 80.0%), *S. epidermidis* (10, 71.4%), Klebsiella spp. (6, 66.7%), *E. coli* (11, 64.7%), and *S. aureus* (11, 64.7%). Majority of the isolates, like *Enterobacter* spp. (4, 80.0%), *Citrobacter* spp. (3 60.0%), *E. coli* (10, 58.8%) and *Klebsiella* spp. (4, 44.4%), were screened for ESBL production. *Enterobacter* spp. (4, 80.0%), *E. coli* (8, 47.1%), *Klebsiella* spp. (4, 44.4%), and *Citrobacter* spp. (2, 40.0%), were among the isolates confirmed for ESBL production (Table 6).

Discussion

A total of 80 bacteria of 9 different species were isolated from a total of 120 samples (milk, yoghurt and milk contact surfaces). Of the isolates, 31 (38.8%) were gram-positive and 49 (61.2%) were gram-negative. Similar studies in Ethiopia[16,19,23,24] also isolated many different bacteria in milk. The high prevalence of bacterial species in this study was associated with commercial use of unpasteurized milk, suboptimal hygiene practices, inadequate refrigeration, and lack of appropriate equipment suitable for storing and transporting milk. Microbial contamination in the milk market value chain can be caused by diseased cows, unhygienic milking practices, poor personal hygiene, unhygienic milking utensils and/or equipment, and poor preservation and inadequate supply of drinking water.[14,25–27] In developing countries

Table 4 Antibiotics Resistance Patterns of Bacterial Contamination in Milk, Yoghurt and Milk Contact Surfaces at Debre Berhan Town, Ethiopia, 2022.

Isolates	AMC (30μg)	AMP (30μg)	PEN (30μg)	SXT (30μg)	CIP (5μg)	CAF (30μg)	GEN (10μg)	ERY (15μg)	TET (30μg)	DXT (30μg)	FOX (30μg)	CRO (30μg)	IMP (10μg)	MRP (10μg)	CTX (30μg)	CAZ (30μg)
S. aureus (17)	16(94.1)	17(100.0)	16(94.1)	9(52.9)	8(47.1)	10(58.8)	9(52.9)	11(64.7)	9(52.9)	10(58.8)	9(52.9)	9(52.9)	4(23.5)	4(23.5)	5(29.4)	4(23.5)
S. epidermidis (14)	14(100.0)	13(92.9)	14(100.0)	6(42.9)	7(50.0)	8(57.1)	6(42.9)	9(64.3)	8(57.1)	8(57.1)	7(50.0)	7(50.0)	4(28.6)	3(21.4)	4(28.6)	4(28.6)
E. coli (17)	16(94.1)	17(100.0)	NT	11(64.7)	8(47.1)	9(52.9)	8(47.1)	11(64.7)	10(58.8)	11(64.7)	8(47.1)	8(47.1)	5(29.4)	4(23.5)	6(35.3)	5(29.4)
Klebsiella spp. (9)	8(88.9)	9(100.0)	NT	6(66.7)	5(55.6)	5(55.6)	4(44.4)	6(66.7)	5(55.6)	5(55.6)	4(44.4)	5(55.6)	3(33.3)	3(33.3)	4(44.4)	4(44.4)
Enterobacter spp. (5)	4(80.0)	5(100.0)	NT	3(60.0)	3(60.0)	4(80.0)	3(60.0)	4(80.0)	3(60.0)	4(80.0)	3(60.0)	3(60.0)	2(40.0)	1(20.0)	2(40.0)	2(40.0)
Salmonella spp. *(7)*	7(100.0)	7(100.0)	NT	4(57.1)	3(42.9)	4(57.1)	4(57.1)	5(100.0)	3(42.9)	3(42.9)	4(57.1)	4(57.1)	0(0.0)	0(0.0)	3(42.9)	3(42.9)
Citrobacter spp. (5)	5(100.0)	5(100.0)	NT	4(80.0)	3(60.0)	3(60.0)	3(60.0)	4(80.0)	3(60.0)	3(60.0)	3(60.0)	3(60.0)	1(20.0)	1(20.0)	2(40.0)	1(20.0)
Proteus spp. (5)	4(80.0)	5(100.0)	NT	3(60.0)	2(40.0)	2(40.0)	3(60.0)	3(60.0)	3(60.0)	3(60.0)	3(60.0)	3(60.0)	0(0.0)	0(0.0)	1(20.0)	1(20.0)
Shigella spp. (1)	1(100.0)	1(100.0)	NT	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Total (80)	75(93.8)	79(98.8)	30(96.8)	46(57.5)	39(48.8)	45(56.3)	40(50.0)	53(66.3)	44(55.0)	47(58.8)	41(51.3)	42(52.5)	19(23.8)	16(20.0)	27(33.8)	24(30.0)

Note: Data are represented as N(%).

Abbreviations: AMC, amoxicillin; AMP, ampicillin; PEN, penicillin; SXT, cotrimoxazole; CIP, ciprofloxacin; CAF, chloramphenicol; GEN, gentamicin; ERY, erythromycin; TET, tetracycline; DXT, doxycycline; FOX, cefoxitin; CRO, ceftriaxone; IMP, imipenem; MRP, meropenem; CTX, cefotaxime; CAZ, ceftazidime; NT, not tested.

https://doi.org/10.2147/IDR.S418793

Table 5 Multiple Drug Resistance Patterns of Bacterial Contamination in Milk, Yoghurt and Milk Contact Surfaces at Debre Berhan Town, Ethiopia, 2022.

Isolates	R0	R1	R2	R3	R4	R5	R6	R7	≥R8
S. aureus (17)	0(0.0)	1(5.9)	3(17.6)	3(17.6)	0(0.0)	3(17.6)	0(0.0)	2(11.8)	5(29.4)
S. epidermidis (14)	0(0.0)	1(7.1)	2(21.4)	1(7.1)	2(21.4)	3(21.4)	1(7.1)	0(0.0)	4(23.5)
E. coli (17)	0(0.0)	1(5.9)	3(17.6)	2(11.8)	3(17.6)	0(0.0)	1(5.9)	2(11.8)	5(29.4)
Klebsiella spp. (9)	0(0.0)	1(11.1)	2(22.2)	3(33.3)	0(0.0)	0(0.0)	1(11.1)	1(11.1)	1(11.1)
Enterobacter spp. (5)	0(0.0)	1(20.0)	0(0.0)	0(0.0)	0(0.0)	1(20.0)	1(20.0)	0(0.0)	2(40.0)
Salmonella spp. (7)	0(0.0)	0(0.0)	3(42.8)	1(14.3)	0(0.0)	1(14.3)	1(14.3)	0(0.0)	1(14.3)
Citrobacter spp. (5)	0(0.0)	0(0.0)	0(0.0)	1(20.0)	1(20.0)	1(20.0)	1(20.0)	0(0.0)	1(20.0)
Proteus spp. (5)	0(0.0)	1(20.0)	2(40.0)	1(20.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(20.0)
Shigella spp. (1)	0(0.0)	0(0.0)	1(100.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Total (80)	**0(0.0)**	**6(7.5)**	**16(20.0)**	**12(15.0)**	**6(7.5)**	**9(11.3)**	**6(7.5)**	**5(6.3)**	**20(25.0)**

Note: Data are represented as N(%).

Abbreviations: R0, not resistant to any antibiotics; R1, resistant to one antibiotic; R2, resistant to two antibiotics; R3, resistant to three antibiotics; R4, resistant to four antibiotics; R5, resistant to five antibiotics; R6, resistant to six or more antibiotics; R7, resistant to seven antibiotics; ≥R8, resistant to eight and more antibiotics.

Table 6 Distribution of MDR and ESBL Confirmed Bacterial Contamination in Milk, Yoghurt and Milk Contact Surfaces, at Debre Berhan Town, Ethiopia, 2022.

Isolates	MDR	Screened for ESBL	ESBL Confirmed
S. aureus (17)	11(64.7)	Not tested	Not tested
S. epidermidis (14)	10(71.4)	Not tested	Not tested
E. coli (17)	11(64.7)	10(58.8)	8(47.1)
Klebsiella spp. (9)	6(66.7)	4(44.4)	4(44.4)
Enterobacter spp. (5)	4(80.0)	4(80.0)	4(80.0)
Salmonella spp. (7)	3(42.8)	3(42.8)	1(14.3)
Citrobacter spp. (5)	5(100.0)	3(60.0)	2(40.0)
Proteus spp. (5)	2(40.0)	1(20.0)	1(20.0)
Shigella spp. (1)	0(0.0)	0(0.0)	0(0.0)
Total (80)	**52(65.0)**	**25(51.0)**	**20(40.8)**

Note: Data are represented as N(%).

like Ethiopia, many dairy farmers do not disinfect the nipples of the cow before milking and wash their hands poorly before milking. Poor hygienic practices and lack of standard milking procedures have been reported throughout the milk value chain system in Ethiopia.[14]

In this study, the *S. aureus* contamination level was 17 (21.3%). This finding is comparable to studies in Tigray, Ethiopia[23] and Sebeta, Central Oromia,[24] but lower than another study in Tigray, Ethiopia.[19] Similarly, this study documented lower levels of contamination compared to the report from Côte d'Ivoire.[28] Furthermore, this study reported a lower rate of *S. aureus* milk contamination than the study in Madurai, South India.[29] High levels of *S. aureus* infection

indicate unhygienic handling and milking procedures. This was further concluded to be due to the predominant presence of *S. aureus* in raw milk (4, 23.5%), yoghurt (5, 29.4%) and milk container swabs (5, 29.4%). The overall contamination rate for *S. epidermidis* was 14 (17.5%). In addition, it was the predominant isolate from milk container swabs (5, 35.7%) and drinking cup swabs (7, 50.0%). This indicates cross-contamination via human skin, as *S. epidermidis* is one of the most common bacterial colonizers of healthy human skin.

This study showed that intestinal bacteria such as *E. coli* (17, 21.3%), *Salmonella* spp. (7, 8.8%) and *Klebsiella* spp. (9, 11.3%) were detected most often. Previous studies have also shown that coliform bacteria are most common in milk and milk containers because they are abundant in animals and in the environment.[30–32] *E. coli* and *Salmonella* spp. presents the main microbiological risks associated with the consumption of dairy products made from raw milk or cow's milk contaminated after pasteurization, mainly in developing countries where hygiene standards are low.[33] Overall, this study reports that milk and dairy products are susceptible to many pathogens, including *Salmonella* spp., *S. aureus* and *Enterobacter* spp., *E. coli* and *Klebsiella* spp., and *Citrobacter* spp. Many factors contribute to the prevalence and presence of pathogens in milk and its products, including farm size, number of livestock, milking hygiene, farm management practices, environmental sanitation for processing, post-processing, and transportation as well as geographical location and season.[9,34–37]

Milk and dairy products are contaminated with AMR pathogens from a variety of sources that pose a risk of contamination, including the animals themselves, dirty milk containers, milk handlers, airborne dust and droplets during production and processing.[37] Therefore, it is very important to minimize the emergence and spread of multidrug-resistant microorganisms that can be transmitted from animals or animal products such as milk and meat, and to maintain the effectiveness of currently available antibiotics.

In this study, the overall resistance of isolates was observed for ampicillin (79, 98.8%), amoxicillin (75, 93.8%) and penicillin (30, 96.8%). For example, *S. aureus* were 100% and 16 (94.1) resistant to ampicillin and penicillin, respectively. Another study also reported the same result for specific bacterial species. For example, a study in Bishoftu, Ethiopia[37] and South Africa[38] reported 100% penicillin resistance rate. *S. epidermidis* also showed 100% and 13 (92.9%) resistance against amoxicillin, penicillin and ampicillin, respectively. Most of the isolates of intestinal bacteria such as *Salmonella* spp., *Citrobacter* spp., *Shigella* spp. also showed 100% resistance to amoxicillin, while *E. coli* and *Klebsiella* spp. were 16 (94.1%) and 8 (88.9%) resistant to amoxicillin, respectively. This may be because these drugs are easily available at low cost leading to abuse and misuse.

This study found relatively high rates of resistance to Ethiopia's most commonly prescribed antibiotics. Examples: 53 (66.3%) on erythromycin, 46 (57.5%) on co-trimoxazole, 47 (58.8%) on doxycycline, 42 (52.5%) on ceftriaxone, 40 (50.0%) on gentamicin and 45 (56.3%) on chloramphenicol were observed. Many studies in Ethiopia and abroad have also reported high levels of individual species resistance to these antibiotics.[39–46] This might be due to over-prescription of antibiotics, under-administration by patients, over-use of antibiotics in livestock and farmland, poor infection control in health services, and poor sanitation and hygiene facilities.

Results of this study showed that 58/80 (72.5%) of the isolates were resistant to three or more antibiotics (eg, *S. aureus* 13/17 (76.5%), *E. coli* 13/17 (76.5%), *S. epidermidis* 11/14 (78.6%), *Citrobacter* spp. 100%, *Klebsiella* spp. 6/9 (66.7%), *Salmonella* spp. 4/7 (57.1%), *Enterobacter* spp. 4/5 (80.0%)). A study conducted in Ethiopia reported higher results than our results for *S. aureus*. For example, a study in Bishoftu,[37] Haramaya[47] and Adama[48] reported resistance rates of *S. aureus* of 98.39%, 87.6% and 94.4%, respectively. However, another study conducted in Addis Ababa, Ethiopia[49] and Brazil,[50] reported 45.1% and 64.4% *S. aureus* resistant to three or more antibiotics, respectively. This study also reported a lower rate of resistance (to three or more antibiotics) for *E. coli* (76.5%) than the study conducted in Bishoftu town, Ethiopia[51] reported 92.5%. In addition, studies in South Africa[52] reported at least three types of antibiotic-resistant *E. coli*. In the present study, *Salmonella* spp. was found to be 57.1% resistant to three or more antibiotics, a comparable study in Bishoftu, Ethiopia[37] and higher than the study in Addis Ababa, Ethiopia[46] reported 53.85% and 50%, respectively. But studies in Jimma town, Ethiopia[53] and Kersa District, Jimma Zone, Ethiopia,[54] reported rates of 83.3% and 70%, respectively. According to this study, with the exception of recently introduced antibiotics in Ethiopia such as meropenem, cefotaxime and ceftazidime, all tested antibiotics were the most frequently observed patterns for most bacteria tested.

Of the 80 bacteria isolated, 52/80 (65.0%) were MDRs and 20/49 (40.8%) isolates were confirmed as ESBL producers. Previous studies conducted in different parts of the world reported increased levels of MDR[5,55–64] and ESBL-producing bacteria.[65–69] The higher levels of resistance observed in this study may be due to inappropriate antibiotic use on dairy farms. The results show that the use of these antibiotics is common in the study area. Significant levels of resistance to many drugs pose a public health risk because foodborne outbreaks are difficult to treat, and this group of MDRs in the food supply is a reservoir for resistance genes that can spread. Due to the relatively limited availability and high cost of newly developed drugs, reports of antibiotic resistance rates for relatively cheap and frequently available antibiotics are alarming for low-income communities living in developing countries, such as Ethiopia.

Limitations of the Study

Identification of diarrheagenic *Escherichia coli* strains, enterotoxic strains of *Staphylococcus aureus*, and species identification of the majority of bacterial isolates was not conducted. Furthermore, clonal relationships of the isolates and molecular characterization of multidrug-resistant and extended-spectrum beta-lactamase-positive isolates was not performed. Owing to the limited sample size, statistical associations that could elucidate the correlation between milk processor hygiene practices and multidrug-resistant and extended-spectrum beta-lactamase-positive isolates were not demonstrated.

Conclusion and Recommendation

The levels of milk contamination in this study were high, suggesting the existence of significant health risks to consumers. Milk collected from farmers, milk vendors and cafeterias was significantly associated with higher levels of milk contamination. Milk distributors and cafe owners should apply good hygiene and hygiene practices when handling milk. Use suitable, clean cold chains and containers during transportation; and refrigerate the milk during storage. Government agencies should establish quality and safety standards for commercially produced milk to improve microbiological quality and milk safety.

In this study, the presence of resistant pathogenic bacteria on milk, yogurt, milk containers and drinking cups indicated poor hygiene, which is a major health concern for consumers. Existing research has also clearly shown that enteric bacteria such as *E. coli* and *Salmonella* spp. isolated from human and animal feces can contaminate milk and milk containers due to poor hygiene standards. In addition, high levels of MDR and ESBL-producing bacteria were detected in milk and milk contact surfaces; it also revealed evidence of inappropriate antibiotic use in animals and humans. Therefore, an ongoing program of resistance surveillance should be implemented nationwide.

Ethical Consideration

Ethical approval was obtained from the Debre Brehan University Institutional Review Board [Protocol Number: IRB-003], and formal support was obtained from the Debre Brehan Town, North Shoa Zonal Office. All participants were informed of the purpose of the study. Ultimately, verbal and written consent was obtained from each milk handlers, trader and distributor.

Disclosure

The authors declare that this study was conducted in the absence of any commercial or financial relationships that would be considered a potential conflict of interest.

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