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Research Article

Isolation and Characterization of Gram-Negative Bacterial Species from Pasteurized Dairy Products: Potential Risk to Consumer Health

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Pathogenic bacterial contaminants of dairy products cause economic and human life losses if not destroyed by pasteurization. Gram-negative bacteria are among such major contaminants. Contamination persists because of faulty pasteurization or occurs during postpasteurization processing. Many factors, including presence of asymptomatic healthy carriers, existence of broad-range host pathogens, and resistance of pathogens to ordinary disinfectants, hamper the control of such pathogens. Here, samples of pasteurized dairy products were subjected to enrichment/selective cultures to test for possible *Salmonella* contamination, followed by growth on/in various media to test for phenotypic properties and some virulence characteristics of isolates (catalase, urease, oxidase, gelatinase, etc.). Isolates were characterized by phenotypic and genotypic tests for identification and resistance to clinically relevant antibiotics, including disk diffusion and for β-lactamase production. All milk samples harbored Gram-negative bacilli, which constitute a public health hazard. All of the isolates exhibited intermediate-level or higher resistance to ≥2 clinically relevant antibiotics, while some were susceptible. None tested positive for phenotypic gelatin hydrolysis but exhibited alpha- or beta-hemolysis. Sequence alignments of 16S rRNA gene partial sequences suggested up to 99% sequence similarities to subspecies of *Salmonella enterica*. Most isolates were also β-lactamase producers, especially *bla*TEM. In conclusion, high contamination rates were found in all Ethiopian pasteurized milk samples. The reasons for this burden of contamination need to be elucidated for meaningful and targeted control. Larger studies are needed, specifically to reveal points of entry of potential pathogens into dairy products. Information from this work will help to address and control previously unrecognized health hazards associated with consumption of pasteurized dairy products.

1. Introduction

Members of the family Enterobacteriaceae are major causes of foodborne infections worldwide. The Enterobacteriaceae are Gram-negative, non-spore-forming, facultatively anaerobic, catalase-positive, oxidase-negative, and generally capable of growth on medium containing simple nutritional substrates as they can ferment a wide range of carbohydrates. This family includes *Escherichia coli*, *Shigella* spp., and *Salmonellae*, among others. While many are part of the normal flora of humans and animals, others such as *Salmonella typhi* are always associated with disease. Others can be occasionally pathogenic—either opportunistically or following acquisition of virulence factors [1]. The Enterobacteriaceae possess complex antigenic structures and produce a variety of toxins and other virulence factors. Humans acquire these bacteria after consumption of contaminated foods, from contact with animals, or directly via human-to-human transmission (e.g., the human-host-specific pathogens such as *S. typhi*) [2]. Contaminated foods of dairy origin account for a large share (14%) of the burden of foodborne illnesses [3]. A current review identified prevalence rates of 6, 9, and 10% of *Salmonella*,

L. monocytogenes, and *E. coli* O157:H7, respectively, in raw cow milk in Ethiopia [4].

The epidemiology of foodborne infections is changing, including its several manifestations: emergence of new and more virulent pathogenic bacterial clades, regional or global spread, and dissemination of antibiotic resistance [5, 6]. Among the foremost virulence factors of Enterobacteriaceae are harborage of plasmid- and/or chromosome-encoded multiple antibiotic resistance traits that can confer difficult-to-treat infectious diseases and/or pose risk of transfer of these traits to other members of the microbiota. Enterobacteriaceae are notorious for their expression of several antibiotic resistances, such as the extended-spectrum β-lactamases.

Nontyphoidal *Salmonella* (NTS), such as *Salmonella typhimurium* and *Salmonella enteritidis*, may be characterized by genomic evolution within the host or during host-to-host transmission, driven primarily by prevailing conditions (e.g., changes in host immunity and presence of antimicrobials) [7]. Several reviews indicated that about 30% of invasive nonmalarial blood stream infections in both adults and children (primarily in the immune-compromised and malnourished) presenting with fever in sub-Saharan Africa are caused by *Salmonella* species, and most of these salmonellosis infections are due to NTS, with case fatality rates of 20–25% [8–10]. The latter study also showed that NTS infections in Ethiopia account for up to 10% of blood stream infections.

Elsewhere, reports indicate infectious diseases due to enteric bacterial pathogens from pasteurized dairy products, e.g., [3, 11, 12]. However, information or research findings from one region may not be directly extrapolated to other areas. Technically, it can be challenging to conduct such studies, and an especially daunting task is tracing the cause-effect connections of foods implicated in such infections, which require conducting controlled, resource- and technology-intensive investigations. These scenarios make prevention of the more feasible approach (both technically and economically) to control the infections, but some data are needed on which actions can be based. Information on foodborne infections of dairy origin in Ethiopia from the points of view of antibiotic resistance and virulence traits is either lacking or scarce. Moreover, there is widespread misunderstanding that pasteurized dairy products are safe for consumption. This small-scale study was undertaken to test for the presence of Gram-negative bacteria in pasteurized milk that can provide some information on the possible health risks from consumption of local pasteurized dairy products by the general public. Herein is reported the isolation and partial characterization of Gram-negative bacteria which were tested for the presence of some virulence and antibiotic resistance traits.

2. Materials and Methods

2.1. The Study Site. Addis Ababa is the capital of Ethiopia. The city's milk supplies come from private dairy industries and dairy cooperatives. Milk pooling is the rule. The same brands of dairy products from the major milk suppliers, all of which have been sampled here, are distributed for sale at all supermarkets of the city. The population size is estimated at 3-4 million. Moreover, it is an international place, hosting more than 125 country embassies and being the seat of many continental and global organizations.

2.2. Samples and Culture Media. Samples of eleven milk products, produced by different private dairies, were purchased from food supermarkets. All samples analyzed in this study were declared to have been pasteurized, or derived from pasteurized products, by the producers. The products were kept refrigerated in the supermarkets. The samples were at ≥3 days before their expiration dates (according to package labels) at the time of purchase. All samples were subjected to preenrichment cultures within one hr of purchase. All broth and agar media, materials, and reagents are from Becton Dickinson (Belgium), unless stated otherwise.

2.3. Culture and Isolation. Samples were inoculated into buffered peptone water (BPW) (HiMedia, India) (10 mL or gram of sample in 90 mL BPW) and incubated for 24 hrs at 37°C. Then, 1 mL of each was inoculated into 9 mL selenite broth and Rappaport-Vassiliadis broth and incubated for 24 hrs at 37°C and 42°C, respectively. Following this, successive 10-fold dilutions were made for each sample and 30 μL was inoculated onto *Salmonella-Shigella* (SS) and xylose lysine deoxycholate (XLD) agars and incubated at 37°C for 24 hrs. Single colonies were picked, restreaked on fresh agar, and incubated at 37°C for 24 hrs. Samples were also inoculated onto MacConkey agar. Then, single colonies were randomly picked and inoculated into Luria-Bertani (LB) broth and grown aerobically at 37°C for 24 hrs. Finally, broth cultures were pelleted, and the pellets were stored in LB broth containing 15% glycerol in multiple aliquots to serve as stocks for subsequent studies. The bacterial loads of randomly selected milk samples were estimated by the most probable number (MPN) method.

2.4. Identification. A combination of cultural, biochemical, and molecular tests was used for identification. Each stored sample was grown on LB agar and Gram-stained. The cultural and biochemical tests were conducted three independent times.

Catalase test was conducted by placing colonies grown on LB agar for 24 hrs on slides and adding 1-2 drops of 30% hydrogen peroxide (H_2O_2).

For motility test, screw-cap tubes (13 by 100 mm) containing 5 mL of sterilized motility test medium were inoculated in the center to a depth of 0.5 inches with a colony grown for 24 hrs. The tubes were then incubated at 35°C or at room temperature for 24 hrs.

For urease test, urea broth was prepared and filter-sterilized through 0.45 μm pore size filters. Three mL volumes were then dispensed into 13 by 100 mm sterile screw-cap tubes. Each isolate was inoculated into the broth using a sterile loop and incubated in water bath set to 35°C, or stored at room temperature, with observations made after 1 hr, 2 hrs, and 24 hrs.

To conduct citrate utilization test, Simon's citrate agar (Oxoid, London, England) was freshly prepared in glass tubes, sterilized, and placed at an angle to form a butt and slant. Each tube of medium was inoculated with 20 hr-grown colony of the dairy isolates and the tubes were loosely capped and incubated at 35°C for 24 hrs or longer, with daily observation of changes in color of the media.

Indole production was tested by inoculating 5 mL of tryptose broth (Oxoid) with a pure colony, incubating at 37°C for 24 hrs, and adding 2-3 drops of indole reagent.

Gelatin hydrolysis test was conducted by stab-inoculating a colony of each isolate into 4 mL of 3% sterile nutrient gelatin in screw cap glass tubes and incubating at 37°C for 5 days. Then, the tubes were placed in refrigerator for 4 hrs before viewing for gelatinase activity. Uninoculated nutrient gelatin served as negative control.

Hemolysis test was performed by streaking a colony of each isolate on blood agar (Oxoid) containing 5% defibrinated sheep blood and incubating at 37°C for 48 hrs. *Streptococcus pyogenes* served as positive control whereas *E. coli* served as negative control for hemolytic activity.

Additional biochemical tests conducted included the oxidase test, conducted using oxidase test strips (Oxoid, Australia); triple sugar iron agar (TSI) test, conducted using TSI agar (HiMedia, India); and growth pattern on eosin methylene blue (EMB) agar. All tests were conducted following standard procedures.

2.5. DNA Extraction, Polymerase Chain Reaction (PCR), and Sequence Comparisons. DNA was extracted from the isolates. The DNA concentrations were measured using Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA) and were in the range of 540–1200 ng/nL. The DNA were used for amplification and then sequencing of the partial 16S rRNA gene. Primer sequences are listed in Table 1. Each isolate sequence was pairwise blasted with different bacterial 16S rRNA gene partial sequences obtained from the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) database, and multiple sequence alignments (MSA) were made with the MSA tool T-Coffee (EMBL-EBI, Hinxton, Cambridge).

PCR was also run using *phoP* and *Hin* primers (Table 1) (*phoP* regulates the expression of genes involved in virulence and macrophage survival of *Salmonella* species and is present in several enteric bacteria including *Salmonella* while *Hin* controls phase variation and is present only in *Salmonella*). These primers and cycling conditions were adopted from Way et al. [13]. The products were run on 1.6% agarose gel, and images were taken using a Bio-Rad Gel Doc UV Trans-Illuminator (Bio-Rad Laboratories, Bio-Rad, USA).

2.6. Antibiotic Sensitivity Tests (ASTs). ASTs to several clinically relevant and available antibiotics were conducted by disk diffusion, and guidelines of the Clinical Laboratory Standards Institute (CLSI, MD, USA) [14] were used. Briefly, broth cultures of the isolates were grown until culture turbidity matched the McFarland 0.5 standard. Sterilized Mueller–Hinton agar was poured into 100 mm plates (20 mL/plate) and when firmly solidified were heavily streaked over the entire agar surface with sterile cotton swab that had been dipped into the bacterial broth culture. Antibiotic disks (Abtek, England), 4 per 13×100 mm plate, were dispensed onto the agar surface after the agar surface has dried (~15 minutes after the streaking). The plates were incubated at 35°C for 24 hrs. The following antibiotic disks were used: chloramphenicol (Chl), 30 μg; tetracycline (Tet), 30 μg; gentamycin (G), 10 μg; ciprofloxacin (Cip), 5 μg; nitrofurantoin (N), 300 μg; ampicillin (Amp), 10 μg; erythromycin (E), 15 μg; trimethoprim-sulfamethoxazole (TMS), 25 μg; amoxicillin/clavulanate (A/C), 20/10; and ceftriaxone (Cef), 30 μg. Inhibition zone diameters were measured in millimeters. Tests were also conducted for β-lactamase production by the isolates using multiplex PCR with primers for extended-spectrum β-lactamases (TEM, CTX-M, and SHV) (Table 1) [15].

3. Quality Control

Escherichia coli (American-type culture collection [ATCC] 25922), *Staphylococcus aureus* ATCC 25923, *Proteus mirabilis* ATCC 29906, *Klebsiella pneumonia* ATCC700603, *Salmonella enteritidis* ATCC 13076, *Pseudomonas aeruginosa* ATCC 27853, *Streptococcus pyogenes* ATCC19615, and uninoculated medium were used as positive or negative controls as appropriate in the cultural, biochemical, and AST tests. Each experiment was repeated 3 times, except the disk diffusion AST, which was tested once.

4. Ethics Statement

This work did not use any human or animal subjects and ethical approval was not required.

5. Results

All pasteurized milk samples from all producers gave growth. All isolates were Gram-negative rods. The milk samples randomly chosen and analyzed for bacterial load contained variable estimated numbers of bacteria. Most were contaminated to very high levels, but milk samples 1 and 9 contained much fewer numbers, indicating product-to-product differences in bacterial loads (Table 2).

Cultural and biochemical test results are shown in Table 3. Colonies did not show blackening on SS or XLD agars, but were rather yellowish. Most of the isolates were hydrogen sulfide negative, but all of the isolates were catalase-positive and oxidase-negative. On EMB agar, all isolates exhibited purple-colored colonies. The control strains and negative control cultures (uninoculated) all showed the typical results for the respective biochemical tests.

The results of the *phoP*/*Hin* duplex PCR reactions showed that most isolates of this study do not belong to the *Salmonella* genus. However, two isolates (6 and 7) were found to be in the coliform group as they tested positive only for the *phoP* gene amplification product (299 bp) (Figure 1, lanes 7 and 5, upper and lower panels, respectively). Isolates

Table 1: PCR primer sequences and their targets used in this work.

Target gene	Primer sequence (5′⟶3′)	Product size (bp)	Reference (s)
Variable regions of 16S rRNA gene (V1–V9)			
27F	AGA GTT TGA TCC TGG CTC AG	1500	Universal primers
1492R	GGT TAC CTT GTT ACG ACT T		
Coliform/*Salmonella*			
phoP	ATGCAAAGCCCGACCATGACG GTATCGACCACCACGATGGTT	299	13
Hin	CTAGTGCAAATT-GTGACCGCA CCCCATCGCGCTACTGGTATC	236	13
β-Lactamases			
$_{bla}$TEM	TCGCCGCATACACTATTCTCAGAATGAC CAGCAATAAACCAGCCAGCCGGAAG	422	15
$_{bla}$CTX-M	ATGTGCAGYACCAGTAARGTKATGGC GGTRAARTARGTSACCAGAAYCAGCGG	590	15
$_{bla}$SHV	TGTATTATCTC(C/T)CTGTTAGCC(A/G)CCCTG GTCCTGCTTTGTTATTCGGGCCAAGC	739	15

Table 2: Estimated numbers of Gram-negative bacilli in some of the tested milk samples.

Sample/isolate	0.1	0.01	0.001	MPN	95% confidence range	Estimated number (mL^{-1})
1	0	0	0	<3	0–9.5	$\sim 3 \times 10^2$
3	3	2	2	210	40–430	2.1×10^6
4	3	3	3	>1100	420–4000	$>1.1 \times 10^{11}$
9	0	0	1	3	0.15–9.6	3×10^3
15	3	3	1	460	90–2000	4.6×10^6
20	3	3	3	>1100	420–4000	$>1.1 \times 10^8$

Table 3: Summary of the cultural and biochemical test results for dairy isolates used in this study.

Test	Isolate										
	1	3	4	5	6	7	9	12	15	20	24
Gram stain	−	−	−	−	−	−	−	−	−	−	−
Catalase	+	+	+	+	+	+	+	+	+	+	+
Motility											
37°C	m	m	nm	m	m	nm	m	m	m	m	nm
RT	m	m	nm	m	m	nm	m	m	m	m	nm
Urease	+	+	−	−	−	−	−	+	+	−	−
Citrate	+	+	+	+	+	+	+	+	+	+	+
Indole production	−	−	−	−	−	−	−	−	−	−	−
Triple sugar iron agar											
Butt	Y	Y	D	Y	Y	Y	Y	Y	Y	Y	Y
Slant	Y	Y	D	Y	Y	Y	Y	Y	Y	Y	Y
Gas	+	+	+	−	+	−	+	+	+	+	+
H_2S	−	−	+	−	−	−	−	−	−	−	−
EMB agar	P	P	P	P	P	P	P	P	P	P	P
MacConkey	Ys	Ys	Ys	Ys	Ys	Ys	Ys	Ys	Ys	Ys	Ys
Oxidase	−	−	−	−	−	−	−	−	−	−	−
Gelatin hydrolysis	−	−	−	−	−	−	−	−	−	−	−
BA	α	β	β	β	α	β	α	α	α	α	β

RT: room temperature, −: negative, +: positive, m: motile, nm: nonmotile, Y: yellow, D: dark, P: purple, Ys: yellowish, EMB: eosin methylene blue, and BA: blood agar (hemolysis).

3 and 12 were also in the coliform group as they showed bands on the gel (both with the 299 bp band) (data not shown). Isolate 1 exhibited both the 299 and 236 bp bands, confirming it is *Salmonella* (Figure 1, lane 8, lower panel). The remaining isolates did not give amplification products with the primers. *S. enteritidis* ATCC 13076, used as positive control in the test for presence of both genes, exhibited both the 299 and 236 bp bands (Figure 1, lane 2 in upper and lower panels).

In the disk diffusion test, almost all isolates were found to be susceptible to both Tet and Chl. The exception is isolate 3, found to be resistant to five major antibiotics: Tet, Chl, Cip, Amp, and Cef. Isolate 3 is especially notorious, with no zone of inhibition at all by Tet and Chl. It was also resistant to Cip, Cef, and Amp.

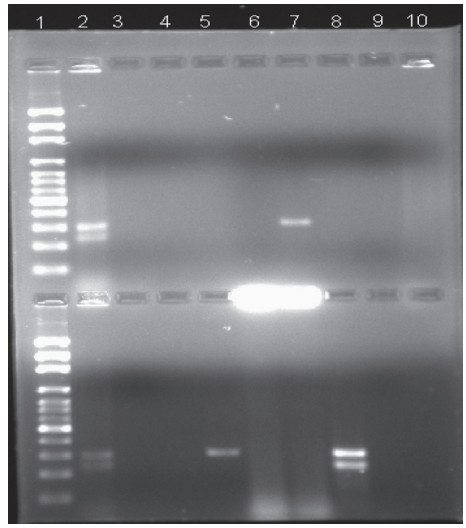

Figure 1: PCR amplification results with *phoP* and *Hin* primers pairs. Upper panel: lane 1: 100 bp molecular weight marker, lane 2: (++)-*S. enterica* ATCC 13076, lane 7: (+) (isolate 6), and lane 10: negative control (no template). Lower panel: lane 1: 100 bp molecular weight marker, lane 2:(++)-*S. enterica* ATCC 13076, lane 5: (+) isolate 7, and lane 8 (++): isolate 1.

The Amp resistance seems to be in line with this isolate's displaying both the 422 and 739 TEM and SHV bands in the ESBL detection test (Figure 2). All of the rest of the isolates were intermediate-resistant to Cip, and all isolates were resistant to Amp (Table 4). All isolates were resistant to E. This is an intrinsic property of members of the *Enterobacteriaceae*, and this test was carried out to check this resistance case. Five of the isolates were multiple drug-resistant to two or more antibiotics.

With respect to β-lactamase, isolate 1 (upper lane 3, Figure 2) was positive for both CTX-M and TEM with 590 and 422 bp bands. Isolate 3 (upper lane 4, Figure 2) was positive for the 422 bp TEM band. Isolate 4 (upper lane 5, Figure 2) was positive for both SHV and TEM bands, indicating the presence of β-lactamases SHV and TEM. Isolates 5, 6, 7, 9, and 12 (upper lanes 6–10) and 15, 20, and 24 (lower lanes 3–5) in Figure 2 were all positive for 422 bp TEM band. Thus, all 11 isolates exhibited the TEM band.

The results of pairwise alignment of partial 16S rRNA gene sequences of the isolates with sequences of subspecies of *S.* enterica showed that the similarities were mostly in the 95 to 99% range (Table 5). Overall, isolate 1 showed the highest nucleotide similarity to the different *S. enterica* subspecies (98-99%). Conversely, the similarities with most of the non-*Salmonella* sequences were found to be much lower (77–85%) (Table 5). Sequence alignments of the isolates to *Cronobacter sakazakii* ATCC 29544 showed them to be in the lower 90s in percent similarity, while similarities to *Aeromonas* ATCC 7966 were in the upper 80s only. The dairy isolates had no more (or less) nucleotide similarity to each other than they had to the *S. enterica* species, with percent similarities ranging between 91 and 98 (data not shown). A phylogenetic tree is shown in Figure 3.

Though the isolates in this study are all Gram-negatives, the sequences of the isolates were also aligned to several Gram-positive sequences such as *S. aureus*, *Lactococcus lactis*, *Clostridium perfringens*, *Listeria monocytogenes*, etc., mainly because most of these bacteria can also be found as contaminants in milk and to show that the isolates of this study are unrelated and to the Gram-positives since this study aimed at analyzing Gram-negative contamination only.

6. Discussion

Dairy products, including some pasteurized ones that still harbor pathogens, can be sources of infections in humans that can result in illnesses, hospitalizations, and even death. This work found Gram-negative bacteria as food contaminants in all of the analyzed dairy products, with potential virulence properties. What makes these findings important is also the fact that the products were labeled as having been pasteurized, which sends the wrong signal to consumers that these products are safe to consume, and it is a common scene that many consumers purchase these products from shops and consume directly from the sachet. Thus, from the findings of this study, it is reasonable to expect that these isolates constitute hazard to the health of consumers. These isolates may have potential for interspecies transfer of the resistance traits they harbor to other commensals or members of the human host gut microbiota [16–20]. Thus, the antibiotic resistance property alone is enough to pose such hazard—whether or not these isolates turn out to be virulent by other mechanisms. Knowledge of virulence and antibiotic resistance factors harbored in microflora of food items, when adequately supported with subsequent appropriate authoritative control measures, is critically important from a public health point of view.

The extent of disease in humans due to milk-borne pathogens is not clearly defined in Ethiopia. The situation in sub-Saharan Africa appears to be serious, as there is frequent exposure to microbe-laden foods. As in other cases, misdiagnosis of cause-effect relationships in disease outcomes is a possibility here. Let alone pasteurized milk, there is also resistance among the public to recommendations to not consume unpasteurized dairy products because of the

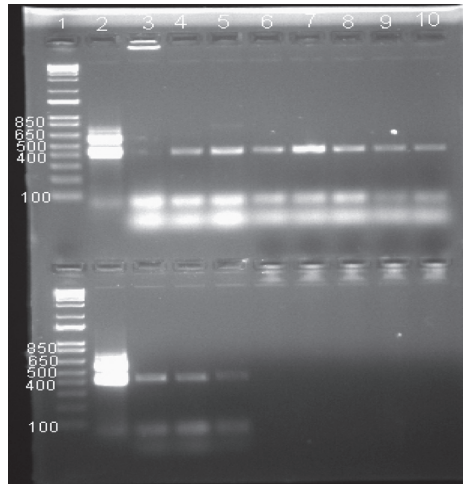

Figure 2: Multiplex PCR amplification results for detection of β-lactamase genes. Upper panel: lane 1: 1 Kb plus molecular weight marker; lane 2: positive control for SHV, CTX-M, and TEM with 739, 590, and 422 bp bands, respectively; lane 3: isolate 1 positive for both CTX-M and TEM with 590 and 422 bp bands; lane 4: isolate 3, positive for TEM (422 bp); lane 5: isolate 4, positive for both SHV and TEM with 739 and 422 bp; lanes 6–10: isolates 5, 6, 7, 9, and 12 all positive for TEM (422 bp). Lower panel: lanes 1 and 2: as in upper panel; lanes 3–5: isolates 15, 20, and 24 all positive for TEM (422 bp).

misconception about the health benefits of raw milk [21, 22]. Meanwhile, the infections (and the probable ensuing diseases) continue to spread, which can be even more disabling when antibiotic resistance is added to the equation.

From the outset, it is possible to surmise a primary reason for the fact that all tested milk samples contained Gram-negative bacteria may be due to the milk pooling practice, where even a single contaminated sample can render the whole pooled milk adulterated. Another likely explanation for the finding of Gram-negative bacteria in pasteurized dairy products is defective pasteurization. Other alternative explanations also exist, such as contamination of the products during postpasteurization handling, or storage at inappropriate temperatures [23, 24]. Psychrotolerant Gram-negative organisms (coliforms or other) can contaminate milk after pasteurization, and though their initial numbers may be low, they grow to predominance during storage at refrigeration temperatures [23, 24]. At this point in this study, it is difficult to say whether the pasteurization processes were less than optimal, or which of these possibilities could be explanation(s) for the present findings; thus, this calls for authoritative investigations.

The cultural and some of the biochemical tests turned out results compatible with those for *Salmonella*. However, the TSI test results were difficult to interpret for some isolates—not fitting entirely into known descriptions of *Salmonella* TSI metabolism, as was the absence of hydrogen sulfide (H_2S) production by most of the isolates. It is possible that the isolates are truly H_2S-negative, considering the yellowish nature of the slants or butts. H_2S-negative *Salmonellae* have been reported, e.g., in [25–27]. Variations in their biochemical properties are not to be unexpected, considering variations in both the diversity and sources of the isolates. Similarly, motility-defective strains are known to exist. For example, aflagellate or flagellar mutants of *Salmonella* occur, with reduced ability in vivo to invade deeper tissue or invade other organs such as the liver and spleen [28, 29].

The MacConkey cultures showed that the isolates were non-lactose fermenters, but that they grew on it (and on SS agar) also showed that they were bile resistant. This can be an important virulence mechanism. The underlying mechanism(s) for this bile resistance capacity was not examined here. However, enteric pathogens can both resist bile and use it as a signal mechanism to induce gene expression to enhance virulence, infection, and biofilm formation [30–32]. Other virulence properties can be envisaged to occur from the information obtained in this work. For example, hemolysis due to hemolysins can damage membranes and erythrocytes [33, 34]; catalase activity endows pathogenic bacteria with resistance to oxidative stress due to hydrogen peroxide that can be encountered in vivo [35]. Thus, these isolates themselves may turn out to be virulent by these and other mechanisms. The resistance of the isolates to several clinically relevant antibiotics is also a serious concern.

The 16S rRNA gene, which is about 1500 bp long, is known to be conserved, with variable sequences interspersed in between. These features make it ideal target for amplification and sequence comparisons for species-level identification of clinically important bacteria [36, 37]. However, there is no consensus on the use of this sequence in phylogenetic analyses. On the other hand, it has been suggested that even the first 500 bp sequences of the 16S rRNA gene can give similar species-level discriminatory power as the entire 1500 bp sequence in more than 90% of comparisons in a given sequence; and the entire 1500 bp sequence can give similar phylogeny as the whole genome [38, 39]. For comparisons involving the 16S rRNA gene, sequence similarity scores of ≥99% and ≥97% were suggested to give species- and genus-level distinctions, respectively [40–42]. Similar levels of sequence identity were obtained in this work to *Salmonella* species for several of the isolates of this study,

Table 4: Antibiotic disk diffusion sensitivity test results.

Isolate	Antibiotic									
	E	Tet	G	Chl	Cip	N	Amp	A/C	TMS	Cef
E. coli®	R	S	S#	R	S	S	R		S	I
S. aureus®®	R	S	S#	S	S	S	R	S	S	I
1	R	S	R	S	I	S	R	ND	ND	ND
3	R	R•	S#	R•	R	S	R•	S	S	R
4	R	ND	ND	ND	ND	ND	ND	ND	S	R
5	R	S	R	S	I	S	R	R	S	I
6	R	S	S#	S	I	S	R	R	R	R
7	R	S	I	S	I	S	R	R	S	I
9	R	S	S#	S	I	S	R	ND	ND	ND
12	R	S	I	S	I	S	R	I	S	S
14◆	R	S	R	S	I	S	R	R	I	R
15	R	S	S#	S	I	S	R	I	S	R
20	R	S	I	S	I	S	R	ND	ND	ND
24	R	S	S#	S	I	S	R	ND	ND	ND

E: erythromycin, Tet: tetracycline, G: gentamycin, Chl: chloramphenicol, Cip: ciprofloxacin, N: nitrofurantoin, Amp: ampicillin, A/C: amoxicillin/clavulanate, TMS: trimethoprim-sulfamethoxazole, Cef: ceftriaxone, ND: not determined, R: resistant, S: susceptible, and I: intermediate. Inhibition zone diameters were measured in millimeters. ®(ATCC 25922); ®®(ATCC 25923). ◆Isolate 14 is from the same source as isolate 1. •Highly resistant, no inhibition zone (0.00 mm). #The CLSI guideline states in vitro activity does not correlate with in vivo activity.

Table 5: Sequence similarities in percent of the study isolates to the 16S rRNA gene sequences obtained from the database.

Comparator	Isolates										
	1	3	4	5	6	7	9	12	15	20	24
Salmonella enterica 13311	98	97	93	98	94	98	95	90	96	96	96
S. enterica ATCC 17058	99	98	94	99	95	98	95	93	99	97	98
S. enterica ATCC 13076	98	96	90	95	92	95	93	90	95	95	95
S.enterica 14847	98	97	92	97	94	97	94	90	98	95	98
S. enterica 9386	98	97	91	95	93	95	94	90	95	95	94
S. enterica ATCC 13314	98	97	91	95	93	96	94	90	96	95	94
S. enterica LT2	98	96	92	97	93	97	94		96	95	96
Enterobacter cloaca	99	98	91	96	94	96	94	91	97	97	96
Citrobacter freundii	95	96	91	95	94	96	95	91	97	96	96
Shigella dysenteriae 13313	97	96	90	93	93	94	94	88	94	95	94
Escherichia coli ATCC 25922	97	95	90	93	92	93	93	88	94	95	93
Serratia marcesens	93	95	91	95	94	94	95	88	95	95	94
Yersinia sp. A125 KOH2	92	94	90	94	95	93	96	87	94	94	93
Proteus mirabilis 29906	90	90	93	89	91	89	92	87	90	90	89
Coxiella burnetii	82	83	84	81	82	83	82	NSH	81	83	83
Haemophilus influenzae 33391	85	83	84	84	85	84	84	79	84	84	83
Pseudomonas aeruginosa 50071T	84	85	86	84	85	85	85	NSH	85	84	84
Brucella abortus 009105	84	84	95	82	83	84	84	NSH	84	83	83
Campylobacter helveticus 51209	80	80	79	78	79	80	80	76	81	86	79
Mycobacterium avium 25291	80	78	80	79	79	81	79	NSH	81	79	80
Clostridium botulinum 19397	81	81	82	86	80	81	81	NSH	82	86	80
Listeria monocytogenes 19112	80	80	81	78	80	80	80	NSH	80	NSH	79
Enterococcus faecium 19434	NSH	79	80	NSH	78	79	79	NSH	80	NSH	78
Enterococcus faeca1is 9433	NSH	77	78	NSH	78	77	79	NSH	78	NSH	77
Staphylococcus aureus 25923	79	80	77	79	78	80	79	NSH	80	79	79
Bacillus cereus 10702	80	80	81	78	79	80	80	NSH	80	NSH	80
Bacillus subtilis	79	79	81	NSH	NSH	79	79	NSH	80	NSH	79
Lactococcus lactis AcCh35	NSH	78	79	NSH	77	78	77		78	NSH	77
Lactococcus lactis AcCh67	NSH	78	79	NSH	77	78	77		78	NSH	77

NSH: no substantial similarity.

although as indicated, these cannot be definitive by themselves. A primary factor appears to be the size of the 16S rRNA gene sequence employed in the analysis. Sequences of some of the different subregions of the full-length variable region (V1–V9) provide good results for classifying different bacteria [43, 44], and a few of the variable regions, or short reads, may also provide species-level or genus-level identification [44, 45]. However, the full-length sequence of the

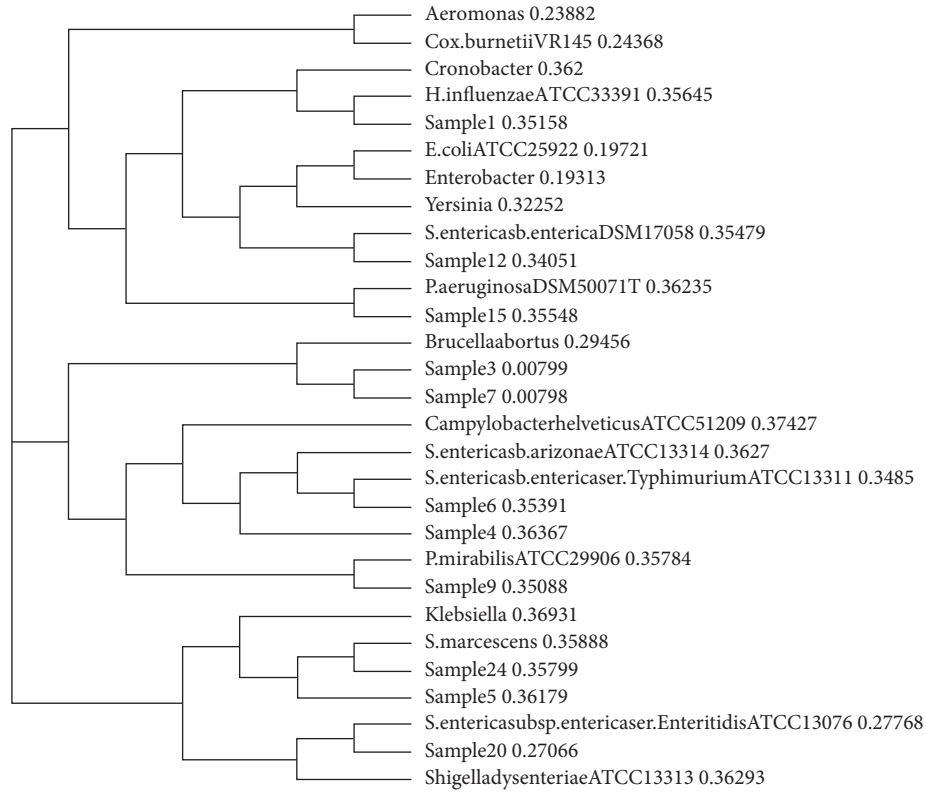

Figure 3: Phylogenetic tree generated with multiple sequence alignment using 16S rRNA gene partial sequences of the study isolates and type strains. "Sample" is synonymous to "isolate" in the text (e.g., sample 1 is the same as isolate 1).

16S rRNA gene with ≥99% sequence identity could better provide species-level resolution [44]. Conversely, the lower the similarity between any two sequences, the more far apart (or unrelated) they should be evolutionally.

This study has some limitations. It will be necessary to investigate the full virulence potentials of the isolates, which was not fully addressed in this work. The isolation and growth conditions in this work were tilted to promote growth of Gram-negative microbes, specifically *Salmonella*, and the possible presence of other microbes cannot be excluded. Definitive identification of the isolates was not achieved in this work; however, this does not diminish the significance of the findings, i.e., the imminent risk from the antibiotic resistance, including transferability, and virulence properties.

7. Conclusions

This study found high levels of possibly pathogenic bacteria possessing antibiotic resistance properties and with also possibility of transfer of antibiotic and virulence properties to gut microflora. With studies on milk-borne pathogens and possible associated health hazards to consumers being scarce in Ethiopia, this study's findings have significance from public health point of view; and importantly, these call for evaluation of both the effectiveness of the pasteurization processes in the dairy plants and inspection of the manners of postpasteurization handling of the products; defects in either one or both of which may explain the reasons for these findings.

Data Availability

Data generated for this study are available from the corresponding author upon request.

Conflicts of Interest

The author declares that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

SHM designed and conducted the experiments, analyzed the data, and wrote the manuscript.

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