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BACTERIOLOGICAL ANALYSIS OF UNTREATED RETAIL RAW MILK COLLECTED FROM RANDOM SUPPLIERS AT DOHUK GOVERNORATE – KURDISTAN REGION – IRAQ

Mohammed Abdul-Wahid Khidhir[1], Fawzi Adil Issa[2*]

[1] Faculty of Science, Dept. of Biology, University of Zakho-Kurdistan Region-Iraq.
[2*] College of Medicine, Dept. of Biomedical Sciences, University of Zakho-Kurdistan Region-Iraq. (fawzi.issa@uoz.edu.krd)

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ABSTRACT

Milk is a high nutritional food and extremely sensitive to bacterial contamination. The current study aimed to assess the presence and density of bacteria in local raw milk. Eighty raw milk samples were collected from four distanced geographical locations at Dohuk Governorate, Kurdistan Region-Iraq. For each geographical site, two private farms were randomly chosen for collecting milk samples. A batch of 10 raw milk samples was obtained from each farm for bacterial availability analysis. All samples were incubated with aeration at 37 °C for 24-48h on specific bacteriological media. Aerobic bacteria were observed in all sheep raw milk samples. The mean counts of total aerobic bacterial in samples from all farms were from 1.0 x 10^4 to more than 3.0 x 10^6 cfu/mL. *Staphylococcus aureus* was found in 37.5% (*n*=30); 50% (*n*=10); for B, D, and K groups, no *S. aureus* was observed in Z group. *S. aureus* density was from 1 x 10^3 to 4.0 x 10^4 cfu/mL (B Group); 2.7 x 10^4 to 3.0 x 10^4 cfu/mL (D Group); and 2.7 x 10^4 to 3.0 x 10^4 cfu/mL (K group). *Escherichia coli* was found in 23.75% (*n*=19); 40% (*n*=8), 50% (*n*=10), and 5% (*n*=1) of the raw milk samples for B, D, and K groups respectively as Z group was free of *E. coli*. *E. coli* contaminated samples produced bacterial growth from 6.0 x 10^3 to 7.6 x 10^4 cfu/mL (B Group); and 1.0 x 10^3 to 6.0 x 10^3 cfu/mL (D group) and only one sample from K group was contaminated with *E. coli* (7.4 x 10^4 cfu/mL). *Klebsiella* spp were observed in 57.5% (*n*=46) of the raw-milk samples; Z group 40% (*n*=8), B group 80% (*n*=16), D group 50% (*n*=10), and K group 60% (*n* =12). Bacterial abundance was from 2.6 x 10^4 to 1.88 x 10^5 cfu/mL (Z group); 1.3 x 10^4 to 1.51 x 10^5 cfu/mL (B group); 6.0 x 10^3 to 1.8 x 10^4 cfu/mL (D group); and from 2.4 x 10^5 to 1.24 x 10^6 cfu/mL (K group). *Shigella* raw milk positive samples were observed in 48.75% (*n*=39); Z group 100% (*n*=20), B group 45% (*n*=9), D group 50% (*n*=10), while K group was free of *Shigella* spp. Bacterial density was from 1.9 x 10^4 to 2.37 x 10^5 cfu/ mL (Z group), from 5.0 x 10^3 to 4.8 x 10^4 cfu/ mL (B group), and from 5.0 x 10^3 to 2.3 x10^4 (D group). All sheep raw-milk samples of this work were completely free of any species of *Salmonella* rods. However, 72 out of 80 examined samples of this study exceeded the total aerobic bacterial count according to the European recommended standards. Good hygienic practices, transporting milk in cold and clean containers, and regular medical checkup for sheep are suggested.

Key words: Milk, Raw-milk, Milk-quality, Hygiene, Pathogens, Bacteriological analysis.

INTRODUCTION

Milk is a high-containing nutritional material liquid and considered to be the main and only primary serving for the mammals' new-borne (Boquien, 2018; Miller *et al*., 2019). Raw milk is rich in many valuable components, like; proteins and lactose in addition to colostrum which boosts the immune system via antibodies (Van Winckel, *et al*., 2011). Different factors determine the milk components such as the individual animal, breed conditions, phase of lactation, age and health status (Magan *et al*., 2021).

Psychrotrophs, non-spore-forming mesophilic and thermophilic bacteria were reported to have potential effects on raw milk spoilage and dairy products contamination (Sadiq *et al*., 2016). Hence, yeasts, molds and a wide-spectrum of bacteria rapidly grow in milk especially at temperature above 16°C (Machado *et al*., 2017). Raw milk is considerably contaminated in a short-time at temperature 37 °C. Like other bacterial growth media, most milk contaminants prefer this temperature for best growth and optimal metabolic activities (Knight-Jones *et al*., 2016). Therefore, milk and dairy producers apply the temperature-control application to prevent any milk spoilage during prolonged storage (Myer *et al*., 2016). Different sources have been confirmed for the bacterial entering into milk, like; animal udder, air, feedstuff, milking equipment, milk storage containers and milking employees (Yuan *et al*., 2019).

Milk usually stored at 4°C, however, psychrotropic bacteria have the ability to multiply at 7 °C or below regardless of their ideal growth temperature (Hilgarth *et al*., 2017). Psychrotrophs typically account near 10% of the microflora present in raw milk, nonetheless, they become predominant during the milk transportation and extended storage at low temperatures (Sorhaug and Stepaniak, 1997). Natural pH and nutrients richness value of raw milk and milk-products provide ideal circumstances for microbial growth. Therefore, detection of different bacterial and fungal species in raw-milk is familiar (Quigley *et al*., 2013). The most predominant psychrotrophic bacterial genera in raw milk were found to be: *Pseudomonas* (Marchand *et al*., 2009), *Chryseobacterium* (Yuan *et al*., 2018), *Serratia* (Machado *et al*., 2017), *Acinetobacter* (Saad *et al*., 2018), and *Flavobacterium* (Stepaniak, 2002).

* Corresponding author

Lactococcus lactis and *L. cremoris* are naturally present in the raw milk and not accounted as milk-contaminants. A group of 20 *L. lactis* strains and 10 biovar of *L. cremoris* have been detected in raw milk (Bayjanov *et al*., 2009; Fernandez *et al*., 2011). However, through milking, milk-transportation, -storage, and -processes, many mesophilic pathogens, like; *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* spp, *Campylobacter* spp. (Quigley *et al*., 2013; Cerva *et al.*, 2014) as well as *Staphylococcus aureus* (Makita *et al*., 2012) have been considered as the milk spoilage causative agents and the main microbiological hazards associated with raw milk consumption (Claeys *et al*., 2013).

Bacterial-milk spoilage occurs via bacterial production of different extracellular heat-stable enzymes (HSE) which remain active even through all milk-processes (Vithanage *et al*., 2016) and lead to poor dairy products quality (Sadiq *et al*., 2016). Protease enzymes hydrolyze milk associated proteins and cause undesirable biochemical changes and un-preferable smell and taste of milk in addition to reducing in the milk shelf-life (Stoeckel *et al*., 2016). Proteases change the characteristics of milk to bitter off-flavor, rotten, age gelation and milk coagulation (Rathod *et al*., 2021; Stoeckel *et al*., 2016). Lipase enzymes accelerate the hydrolysis of triglycerides, consequential ultra-heat treatment (UHT) milk rancid, butyric, or even soapy taste, and reducing in milk foaming qualities (Chen *et al*., 2003; Bekker *et al*., 2016). Phospholipases enzymes decline integrity of the milk fat globule membrane, allowing milk's endogenous lipases to greater lipolysis (Lilbeak *et al*., 2007). Some other HSE like galactosidases play important role in milk spoilage as they catalyze the hydrolysis of -1,4-galactosidic bonds in milk lactose (Deeth *et al*., 2002; Chen *et al*., 2009).

Recently, many milk and dairy related poisoning cases have been reported in Dohuk Governorate - Iraq; cases were featured with stomach pain resulting in abdominal cramping, vomiting and diarrhoea post consumption local milk or dairy products. No hygienic and microbiological investigation studies have been done on the local raw milk. The aim of this study focused on the screening of sanitary and bacteriological aspects of the local raw milk via analysing samples from four local farms.

MATERIALS AND METHODS

The current study was conducted from September 2021 to June 2022 in Dohuk Governorate, Kurdistan Region- Iraq. All experiments mentioned in this work were done in sterile conditions and repeated for two or three times according to the nature of test and the measurements' average were considered.

Collection and Preservation of Milk Samples

Milk samples were collected between September 2021 and January 2022. From each farm, two batches (10 samples for each) were obtained (eighty samples for the whole study). Each one of the 80 samples consisted of 25-400 mL of sheep raw milk directly collected from sheep udders into detergents-washed containers and transferred into sterilized Duran bottles for microbiologically assessment. Bottles were kept with ice cubes in a thermo-isolated boxes and directly addressed to the microbiology laboratory within 1-2 h post-collection or stored overnight at 4ºC prior transferring to the lab.

Classification of Raw-Milk Samples

Samples were collected from four locations: Balcos Village, Zakho City, Khanke Village and Dohuk City, all located in Dohuk Governorate. Each sample was given a code and designed in a "digit-letter-digit" pattern as the first 'digit' refers to the batch number (1 or 2), the 'letter' stands for the first letter of location (Z, B, D, and K), and the last 'digit' refers to the milk sample number (1-10). For instant, "2B7" means the sample number is seven from the batch number two of Balcos collection and so on.

Preparation of Microbiological Media

All bacterial media components were prepared as described by Sambrook *et al*. (2001) or according to the manufacturer instructions. For each medium, up to 1 liter was prepared with desired pH followed by autoclaving.

Preparation of Serial Dilutions

For all raw milk-samples, serial dilution culture-most probable number method was used for samples dilution (Cullen and MacIntyre, 2016). The raw-milk samples were diluted along several dilution factors. 1 mL of milk sample was diluted in 9 mL dH_2O. This was the initial dilution (10^{-1}). To prepare decimal dilutions, 1.0 mL of the (10^{-1}) dilution was transferred to another 9 mL of dH_2O to compose the 10^{-2} dilution. Using a fresh pipette/pipette tip for each dilution, the above step was repeated to produce further decimal dilutions until the suitable bacterial concentration was obtained.

Isolation of Bacteria

One milliliter of suitable dilution of raw-milk was aseptically transferred into sterile Petri-plate. Around 21-23 mL of a specific culture medium was poured onto the diluted milk and mixed well by moving the plate horizontally and gently for 3-6 times left and right.

Identification of Isolates

Gram Staining

Isolates – when required - were addressed to Gram-staining technique to determine the bacterial morphology (Colco, 2005).

Coagulase Test

Coagulase test was performed for the differentiation of *Staphylococcus* spp (Thirunavukkarasu and Rathish, 2014).

Bacterial Isolation Media

Lauria Bertanti Medium

LB medium, (Sigma-Aldrich, UK) was used for stock cultures and isolation of aerobic microorganisms (Medina *et al*., 2011).

Mannitol Salt Agar

MSA, Neogen Corp., USA, (Neogen Corp, 2011) was used to isolate *Staphylococcus* spp. from desired dilution factor of raw milk by pour plate technique (Sharp and Searcy, 2006).

Violet Red Bile Lactose

VRBL agar Himedia, USA, (Van Tassell *et al*., 2011) was applied for the isolation and identification of *Escherichia coli* and *Salmonella* spp.

MacConkey Agar

MAC, Neogen Corp., USA, (Cheng *et al*., 2012) was performed as a first choice for the isolation and identification of *E. coli* and *Klebsiella* spp. For further identification, isolates were sub-cultured on Eosin-Methylene Blue (Merck KGaA, Germany) (Abdullah *et al*., 2012).

Xylose Lysine Deoxycholate Agar

XLD agar, Himedia, USA (Nye *et al*., 2002) was used for the isolation of *Salmonella* spp. and *Shigella* spp. For confirmation of the bacterial identity, bacteria were sub-cultured on the Triple-sugar iron (HiMedia) (Siddiquie and Mishra, 2014).

RESULTS AND DISCUSSION

Isolation of Total Aerobic Bacteria

Depending on appendix III, section IX, Chapter I of Regulation (EC) No 853/2004 of the European Parliament (European Union, 2004a) in addition to Council of 29 April 2004 (European Union, 2004b), total bacterial count (TBC) in raw milk should not exceed 1.0 x 10^5 cfu/mL. Out of 80 raw milk samples, only eight of them (2B1-3, 6, 7, 1K8-10) contained less than 1.0 x 10^5 cfu/mL while all the other samples exceeded the European recommended rate (Table 1).

All samples were analyzed at the level of 10^{-4} dilution factor (DF). Highest colony forming unites (cfu) was found in the D group raw-milk batches, all samples showed bacterial heavy growth (BHG) phenomenon (more than 300 cfu/mL). Lower cfu frequency was demonstrated in the Z group raw-milk; from batch 1; 8 out of 10 produced BHG, the other two samples produced 2.0 x 10^5 (1Z6) and 2.4 x 10^5 (1Z7) cfu/mL. For batch 2, the total bacterial counts (TBC) was from 1.3 x 10^5 (2Z9) to 3.3. x 10^5 (2Z1) cfu/mL, with two BHG (2Z7- and 8) samples (Table 1). Further lower decrease in milk-bacterial density was observed in the K group raw-milk collections; bacterial density was from 2 x 10^4 (1K8) to 1.08 x 10^6 (1K4) cfu/mL for batch 1, in batch 2; TBC ranged from 1.7 x 10^5 (2K3) to 1.08 x 10^6 (2K2) cfu/mL with one BHG (2K1). Lowest TBC growth was found in B group samples; bacterial concentration ranged from 3.2 x 10^5 (1B4) to 1.68 x 10^6 (1B6) cfu/mL in batch 1 and from 1.0 x 10^4 (2B6) to 8.2 x 10^5 (2B5) cfu/mL for batch 2 without any BHG in both batches (Table 1).

Table 1: Isolation of aerobic bacteria from 80 sheep raw-milk samples. Isolation include pathogens and non-pathogens bacteria, bacteria were isolated from one ml of raw-milk on the bacterial inhibitors free LB agar medium by incubation at 37 °C for 48h in aerobic conditions.

Groups	Batch No. DF	Sample codes and corresponding CFU									
Z group	Batch 1 samples	1Z1	1Z2	1Z3	1Z4	1Z5	1Z6	1Z7	1Z8	1Z9	1Z10
	DF (10^{-4})/ cfu	BHG	BHG	BHG	BHG	BHG	20	24	BHG	BHG	BHG
	Batch 2 samples	2Z1	2Z2	2Z3	2Z4	2Z5	2Z6	2Z7	2Z8	2Z9	2Z10
	DF (10^{-4})/ cfu	33	19	20	24	26	16	BHG	BHG	13	17
B group	Batch 1 samples	1B1	1B2	1B3	1B4	1B5	1B6	1B7	1B8	1B9	1B10
	DF (10^{-4})/ cfu	92	116	60	32	60	168	148	88	64	112
	Batch 2 samples	2B1	2B2	2B3	2B4	2B5	2B6	2B7	2B8	2B9	2B10
	DF (10^{-4})/ cfu	10	10	3	19	82	1	9	17	14	21
D group	Batch 1 samples	1D1	1D2	1D3	1D4	1D5	1D6	1D7	1D8	1D9	1D10
	DF (10^{-4})/ cfu	BHG	BHG	BHG	BHG	BHG	BHG	BHG	BHG	BGH	BGH
	Batch 2 samples	2D1	2D2	2D3	2D4	2D5	2D6	2D7	2D8	2D9	2D10
	DF (10^{-4})/ cfu	BHG	BHG	BHG	BHG	BHG	BHG	BHG	BHG	BHG	BHG
K group	Batch 1 samples	1K1	1K2	1K3	1K4	1K5	1K6	1K7	1K8	1K9	1K10
	DF (10^{-4})/ cfu	28	32	56	108	64	64	48	2	9	7
	Batch 2 samples	2K1	2K2	2K3	2K4	2K5	2K6	2K7	2K8	2K9	2K10
	DF (10^{-4})/ cfu	BHG	108	17	72	32	88	56	100	120	28

Key: ①Z1 = Batch number, 1Ⓩ1 = Collection point (Zakho), 1Z① = Sample number, B = B group, D = D group, K = group, DF = Dilution Factor, BHG = Bacterial Heavy Grouth (Too many to count – more than 300 cfu/mL).

This work analysis revealed that only 8 (2B1-3, 2B6-7, and 1K8-10) out of 80 (8.75%) samples reached the European recommened bacterial density in untreated milk. However, our findings are in agreement with analysis of previous investigations oucomes which found that exceeding the maximum acceptable level of TBC in raw milk samples is not unusual. For instant, 47 out of 855 raw milk samples were found to exceed the highest reference level of TBC in a study carried out in New York State from 1993 to 1996. The bacterial growth range was from 1.0 x 10^5 to 5.0 x 10^6 cfu/mL (Boor *et al*., 1998). Much higher bacerial contamination levels were noticed in 120 milk samples collected from 3 regions in Sudan between August 2003 and January 2004. Colony forming units in that work were 4.0 x 10^5 to 3.3 x 10^{11}, 1.8 x 10^6 to 1.4 x 10^9, and 5.0 x 10^5 to 5.2 x 10^9 for the three regions, respectively (Ibtisam *et al*., 2007).

A previous study also confirmed the role of season on unpasteurized milk bacterial contamination as the TBC were; 7.7 × 10^5 to 3.3 × 10^{11} cfu/mL in the summer, and 4.0 × 10^5 to 1.4 × 10^9 cfu/mL in the winter confirming that the raw milk is much contamible in summer season compred to winter (Ibtisam *et al*., 2007). Season-associated impacts on the TBC was also noticed in an analyzing of 235 cow milk samples in Prince Edward Islands (Elmoslemany *et al*., 2009), and in a year-round work on 1,144 farms in the Belearic Islands (Soler and Ponsell, 1995). Both of the above studies also confirmed the untreated milk sensitivity to the bacterial mediated milk-spoilage in summer more than in winter. Therefore, the bacterial density could be much lower if this study is repeated in winter due to the session-related effects on the bacterial-associated milk contamination. Furthermore, not all isolated bacteria are pathogens and majority of them will be elemintated through milk precesses.

Detection and enumeration of Staphylococcus aureus

All raw milk samples were bacteriologically analysed using MSA selective medium. At DF of 10^{-3}, no bacterial growth (BG) was found in Z group batches, B group/ batch 2, D group/ batch 2, and K group/ batch 1 (data not included in Table 2). However, *S. aureus* was detected in the samples of B group/ batech 1 in a spectrum of 1.0 x 10^3 (1B7) to 4.0 x 10^4 (1B6). D group/ Batch 1 revealed *S. aureus* BG rate from 2.7 x 10^4 (1D4 and 1D7) to 3.0 x 10^4 (1D1). Finally, *S. aureus* was also observed in K group/ batch 2, BG ranged from 2.7 x 10^4 (2K10) to 3.0 x 10^4 (2K3) (Table 2). Staphylococcal isolates' identification was further established by Gram-stain technique and coagulase test (data not shown).

Table 2: Analysis of *Staphylococcus aureus* presence in 80 sheep raw-milk samples. Pathogen was isolated from 1 ml of raw-milk on selective MSA medium in aerobic conditions at 37 °C for 48h.

Groups	Batch No. DF	Sample codes and corresponding CFU

B group	Batch 1	1B1	1B2	1B3	1B4	1B5	1B6	1B7	1B8	1B9	1B10
	DF (10^{-3})/ cfu	6	24	29	2	3	40	1	20	3	5
D group	Batch 1	1D1	1D2	1D3	1D4	1D5	1D6	1D7	1D8	1D9	1D10
	DF (10^{-3})/ cfu	30	28	29	27	28	28	27	29	29	29
K group	Batch 2	2K1	2K2	2K3	2K4	2K5	2K6	2K7	2K8	2K9	2K10
	DF (10^{-3})/ cfu	28	29	30	29	29	29	29	29	28	27

Key; all details as in table 1.

S. aureus is a serious pathogen because of its wide distribution, high incidence rate, and rapid transmission. It is the causative agent of many clinical problems, from simple superficial skin lesions to hard invasive diseases (Turner *et al*., 2022). Interestingly, no *S aureus* have been detected in all the samples of some batches (1Z*n*, 2Z*n*, 2B*n*, 2D*n*, and 1K*n*), while the samples; 1B*n*, 1D*n* and 2K*n* were *S. aureus* positive. This phenomenon of contamination could be due to different factors shown below; A) the presence of an animal with mastitis (infection of mammary glands) that is the major source of sheep milk contamination (Jayarao *et al*., 2004). B) milking equipment and milkers hands (Cullor, 1997). C) poor applied hygenic standard (Borena *et al*., 2023). D) milking processes and michanisms (Johler *et al*., 2018). Nevertheless, milk pasteurization sgnificatly decreases the number of *S. aureus* cells (Jorgensen *et al*., 2005).

From the whole 80 milk collected samples, 37.5% (n = 30) of the milk samples were *S. aureus* positive, pathogen presence ranged from 1.0 x 10^3 (1B7) to 4.0 x 10^4 (1B6) cfu/mL with a mean value of 2.3 x 10^4 cfu/mL. Fairly, comparable *S. aureus* prevelence rates were obsereved in several studies; Muehlherr *et al* (2003) detected *S. aureus* in 32% in goat and 33% in sheep milk samples in Switzerland. Another study conducted by Vahedi and colleagues revealed a 36% of *S. aureus* prevalence in raw and unpasteurized cow milk in Iran (Vahedi *et al*., 2013). Close *S. aureus* prevalence ratio was found in camel raw-milk samples in Egypt (Elhosseny *et al*., 2018) and in Ethiopia (Tasse *et al*., 2022) where both works reported 38.5%, and in Kenya (Gitao *et al*., 2014) the occurrence of *S. aureus* was around 34.9%. Higher prevelance, 46% of the raw bulk tank milk samples were also noticed to be *S. aureus* positive (Merz *et al*., 2016). The variety of *S. aureus* occurrence in different studies may be due to number of samples, period and time of study, type of milk, and method of investigation. Staphylococcus-mediated infections are responsible for approximately 40.0% of the animal mastitis cases in some countrie (Kateete *et al*., 2013; Basanisi *et al*., 2017) which – in turn – is the main reason of *S. aureus* mediated milk contamination (Li *et al*., 2017).

Detection and Enumeration of E. coli on MacConkey and VRBL Agar

MacConkey agar was used for primary isolation of *E. coli*. Post incubation at 37 °C for 48 h, colonies with pinkish red color and bile precipitate were accounted to be *E. coli* strains (Jorgensen *et al*., 2015). All isolates which produced *E. coli* charateristics were further identified on VRBL agar. All isolates, on VRBL agar, were found to be *E. coli* as they fashioned violet-red colonies with diameter of around 0.5 mm and surrounded by a reddish-fuchsia tight halos resulted from bile salts precipitation confirming lactose decomposition in acid (Leclercq *et al*., 2002). No *E. coli* isolates were observed from both batches of Z group, from batch 2 of B and D groups in addition to the batch 1 of K group (data not shown in Table 3). At DF of 10^{-3}, B group/ batch 1 formed 6.0 x 10^3 (1B6) to 7.6 x 10^4 (1B7) cfu/mL with two bacteria-free samples (1B3-4) (Table 3). Concerning group D, sample 1D6 was contaminated with 6.0 x 10^3 *E. coli* strains while the 9 remaining samples demonstrated only one cfu/mL. With the exception of sample 2K3 that produced 7.4 x 10^4 *E. coli* cella/ mL. All the other remaining sample were free of *E. coli*. Usually, *E. coli* presence in raw milk belongs to the faecal-mediated contamination during milking process (Table 3).

Table 3: Number and percentage of *E. coli* in raw-milk samples. Of all 80 samples, 23.75% were found to be *E. coli* positive. However, 9 out of 19 samples produced only 1 cfu at DF of 10^{-3}. All Z group samples, batch 1 of K group, batch 2 of B and D groups were free of *E. coli*.

Groups	Batch No. DF	Sample codes and corresponding CFU									
B group	DF (10^{-3})	1B1	1B2	1B3	1B4	1B5	1B6	1B7	1B8	1B9	1B10
	Batch 1/ cfu	15	7	0	0	37	6	76	8	9	11
D group	DF (10^{-3})	1D1	1D2	1D3	1D4	1D5	1D6	1D7	1D8	1D9	1D10
	Batch 1/ cfu	1	1	1	1	1	6	1	1	1	1
K group	DF (10^{-3})	2K1	2K2	2K3	2K4	2K5	2K6	2K7	2K8	2K9	2K10
	Batch 2/ cfu	0	0	74	0	0	0	0	0	0	0

Key; all details as in table 1.

E. coli O157 strain has been the target of bacteriological raw mik assessment in several investigation in the last two decates. O157 strain was considerd to be the main causative agent of the milk mediated outbreak (Currie *et al*., 2018; Honish *et al*., 2005; McCollum *et al*., 2012). The presence of *E. coli* is an indicator of potential risk of enteric pathogens in food. The occurrence of *E. coli* is a result of faecal-food contamination where the bacterial loads corresponded with farm hygiene criteria, the condition and effectiveness of cleaning of milking equipment, and the temperature that milk is held at in bulk storage tanks (Leclercq *et al*., 2002). Over all, *E. coli* strains were detected in 23.75% (n = 19) of the untreated milk samples.

The current findings are harmonious with several previous studies; similar finding; 23% was reported from Tigray (Abebe *et al*., 2014), 26% from Ethiopia (Farhan *et al*., 2014), and 23.3 from Egypt (Elbagory *et al*., 2015). The prevalence of *E. coli* in the current study samples were much lower than those found in other works; 44% and 33.9% were reported from Ethiopia by Shunda *et al*. 2013) and Disassa *et al*., (2017) respectively. This relatively high *E. coli* abundance indicated animal health status and their breeding conditions (Vahedi *et al*., 2013). Extreme *E. coli* contaminated raw milk samples were also previously reported; 69% and 63% of milk positive samples were observed in Sudan (Ali and Abdelgadir, 2011) and Tanzania (Lubote *et al*., 2014) respectively. Above high bioavailability number of *E. coli* in Sahara and sub-Sahara countries probably belongs to the bacterial fast growing due to hot climate nature and the absence of cooling systems. However, the finding of this study was higher than those observed in some

conducted studies for example, a study carried out by Lye and colleagues found only 8.75% of *E. coli* positive samples in Malaysia (Ley *et al*., 2013) while Addo *et al*. reported 11.2% from Ghana (Addo *et al*., 2011). Based on all the above investigations, the presence of *E. coli* in unpasteurized milk samples depends on many factors through the milk process from milking and sheep fitness status to milk consumption.

Detection Rate of Klebsiella spp.

The initial isolation of *Klebsiella* spp was performed on MacConkey agar as shown in the above conditions. Colonies charecteristed with large, mucoid, and glistening pink were counted as *Klebsiella* spp (Cheng *et al*., 2021). BG rates varied in all samples, suspected colonies were sub-culctured on Eosin-Methylene Blue (EMB). According to colonies appearnce, all the isolated samples were found to be *Klebsiella* spp by observing bacterial colonies with pink to purple in color without green metallic sheen (Batra, 2018).
Klesiella positive samples were 57.5% (n = 46). Z group/ Batch 2, and D group/ batch 2 were *Klebsiella*-free samples (data not shown in Table 4). No *Klebsiella* spp were detected in two (1Z9-10) out of ten samples of Z group/ batch 1. All the remaining samples produced BG ganged from 2.6 x 10^4 (1Z1) to 1.88 x 10^5 (1Z7) cfu/mL. Six out of ten samples of the B group/ batch 1 contained *Klebsiella* spp producing BG spectrum from 5.2 x 10^4 (1B6) to 7.6 x 10^4 (1B5) at DF of 10^{-3} cfu/mL. All samples of B group/ batch 2 were contaminated with *Klebsiellla* illustrating BG scale from 1.3 x 10^4 (2B9) to 1.51 x 10^5 (2B10) cfu/mL (Table 4).
All the samples of the D group/ batch 1 were *Klebsiella* positive; the presence of this pathogen ranged from 6.0 x 10^3 (1D9) to 1.8 x 10^4 (1D1) cfu/mL. All the samples of the K group/ batch 1 were *Klebsiella* contaminated, and the samples showed bacterial containing ranged from 5.3 x10^4 (1K5) to 1.24 x 10^5 (1K4) cfu/mL at DF 10^{-3}. Eight out of 10 samples of batch 2 were free of *Klebsiella*, the other two samples showed 2.4 x 10^4 (2K4) and 1.05 x 10^5 (2K1) cfu/mL (Table 4).

Table 4: Detection of *Klebsiella* spp in sheep raw-milk samples. *Klebsiella* was found 46 out of 80 samples (57%). *Klebsiella* was initially investigated at DF 10^3. On MacConkey agar and bacterial identity was confirmed on EMB.

Groups	Batch No. DF	Sample codes and corresponding CFU									
Z group	DF(10^{-3})	1Z1	1Z2	1Z3	1Z4	1Z5	1Z6	1Z7	1Z8	1Z9	1Z10
	Batch 1/ cfu	26	104	184	96	112	108	188	172	0	0
B group	DF(10^{-3})	1B1	1B2	1B3	1B4	1B5	1B6	1B7	1B8	1B9	1B10
	Batch 1/ cfu	0	70	0	62	76	52	53	57	0	0
	DF(10^{-3})	2B1	2B2	2B3	2B4	2B5	2B6	2B7	2B8	2B9	2B10
	Batch 2/ cfu	31	40	19	35	56	18	18	20	13	15l
D group	DF(10^{-3})	1D1	1D2	1D3	1D4	1D5	1D6	1D7	1D8	1D9	1D10
	Batch 1/ cfu	18	15	9	16	10	12	12	16	6	11
K group	DF(10^{-3})	1K1	1K2	1K3	1K4	1K5	1K6	1K7	1K8	1K9	1K10
	Batch 1/ cfu	105	89	65	124	53	105	100	105	113	110
	DF(10^{-3})	2K1	2K2	2K3	2K4	2K5	2K6	2K7	2K8	2K9	2K10
	Batch 2/ cfu	105	0	0	24	0	0	0	0	0	0

All details as in table 1.

Isolationn and Identification ofShigella

Shigella spp were isolated on Xylose Lysine Deoxycholate (XLD) agar which is a selective and differential medium used for the isolation of Gram-negative enteric pathogens from fecal specimens, clinical material, food samples, and dairy products (Nye *et al*., 2002). XLD is fundamentlly used for the isolation of *Salmonella* spp. and *Shigella* spp (Maddocks *et al*., 2002). Post incubation on XLD agar at 37 °C for 24 h in aerobic conditions, red colonies were considered to be *Shigella* spp. *Shigella* spp positive samples were 48.75 % (n = 39). B group/ batch 2, D group/ batch 2, K group/ batch 1 and 2 were *Shigella*-free batches DF of 10^3 (data not shown in Table 5). All the samples of Z group were found to be *Shigella* contaminated. BG of Z group/ batch 1 was ranged from 1.9 x 10^4 (1Z5) to 2.37 x 10^5 (1Z8) while it was from 4.8 x 10^4 (2Z3) to 8.9 x 10^4 (2Z10) cfu/mL for Z group/ batch 2. Z group/ batch 1 was more *Shigella* contaminted than the batch 2 with BG mean 7.97 x 10^5 and 6.57 x 10^5 cfu/mL respectivly. One sample (1B8) from B group/batch 1 was free of *Shigella* while the remaining samples produced BG from 5.0 x 10^3 (1B7) to 4.8 x 10^4 (1B3) cfu/mL at DF of 10^{-3}. Finally, all the samples of the D group/ batch1 were noticed to be contaminated with *Shigella* spp producucing BG from 5 x 10^3 (1D2) to 2.3 x 10^4 (1D4) cfu/mL (Table 5), after incubation at 37 ºC for 24h with areation.

Table 5: Isolation of *Shigella* spp from the sheep raw-milk samples. *Shigella* has been observed in 48.75% of the investigated milk samples at DF 10^3. Isolation was involved XLD agar and incubation at 37°C for 24-48h with aeration.

Groups	Batch No. DF	Sample codes and corresponding CFU									
Z group	Batch 1	1Z1	1Z2	1Z3	1Z4	1Z5	1Z6	1Z7	1Z8	1Z9	1Z10
	DF (10^{-3})/cfu	72	38	71	40	19	44	156	237	49	71
	Batch 2	2Z1	2Z2	2Z3	2Z4	2Z5	2Z6	2Z7	2Z8	2Z9	2Z10
	DF (10^{-3})/cfu	72	59	48	61	57	63	68	57	83	89
B group	Batch 1	1B1	1B2	1B3	1B4	1B5	1B6	1B7	1B8	1B9	1B10
	DF (10^{-3})/cfu	20	14	48	16	15	39	5	0	28	47
D group	Batch 1	1D1	1D2	1D3	1D4	1D5	1D6	1D7	1D8	1D9	1D10
	DF (10^{-3})/cfu	16	5	18	19	14	15	14	15	23	18

Key: all details as in table 1.

The prevelance of *Shigella* in raw milk was widely studied worldwide. Some studies focused on the occurance of *Shigella* spp in raw milk and others dealt with the *Shigella* molecular identification in contaminated and unpasteurized milk. Rate of the *Shigella* presence in raw milk is widley unhormonized; a few studies proposed very low percentages such as 3.2% (Gamal *et al*., 2018) and 4.41% (Nisa *et al*., 2021). Relatively, higher density of *Shigella* spp was also demonstrated like 17.5% (Reta *et al*., 2016) and 37% (Thabet and Abd-Eihamid, 2020). However, extrame *Shigella* occurance (80.7%) in raw milk was correspondingly revealed (Oueslati *et al*., 2011). This wide-range of the *Shigella* presence – as in the cases of above pathogens – belongs to all the process of milk production from the milking michanisms to milk consuming.

Private dairy farms are run by the Ministry of Agriculture and Animal Wealth standards and guidance. Dairy producing animal breeding farms are regularly visited by authorized governmental inspection teams to ensure the application of the herd health, breeding conditions, and hygiene procedures. A wide range of veterinary medicines are frequently used to control sheep-related infections such as Oxydone forte 30%, Univet, Tetroxy, LA 20%, Uvemec, Flama-Oxytetra 5%, AnexC-Care etc to decrease the sheep bacterial mediated infections to the minimal levels. Farmers, also, use pre-soap- or pre-detergents-washed stainless steels containers (utensils) for milk collection so as to reduce milk contamination. In spite of the low number of pathogenic and non-pathogenic bacteria in most of the raw-milk samples in this study, it is not recommended to consume raw milk without any heat-treatment as it contains high level of harmful bacteria (Quigley *et al*., 2013). Nevertheless, majority of pathogens are removed or inactivated through an adequate pasteurization technique (Singh and Vyas, 2022).

Only seven out of eighty samples were compatible with the European raw milk standards in containing aerobic bacteria. Exceeding the milk-borne aerobes recommended rates is not unusual aspect in milk quality. Many reasons have been proposed for the aerobes-associated raw milk contamination and spoilage like; teat apex, milking equipment (Coorevits *et al*., 2008); air, water, feed, grass, soil in addition to several environmental conditions (Lejeune and Rajala-Schultz, 2009; Vacheyrou *et al*., 2011). Quantity and diversity of microorganisms in raw-milk do not reflect the quality of milk as many of the growing bacteria are presumed to be non-pathogens (Quigley *et al*., 2013). However, this approach demonstrates the health status of the sheep and hygienic standards that are applied in each farm (Bogdanovicova *et al*., 2016).

Z group raw milk found to be the healthiest milk as it was free of *S. aureus* and *E. coli* pathogens. Furthermore, no *Klebsiella* was observed in Batch 2 while an average of 99 cfu was obtained in Batch 1 samples. Nevertheless, both batches of the Z group were contaminated with *Shigella* spp, by producing cfu means of 88 and 57 per ml at DF of 10^{-3} for batch 1 and 2 respectively (Table 6). Absence of *S. aureus*, *E. coli* and *Klebsiella* (in batch 2) reflects the commitment of this farm with the general official hygienic recommendations which was not noticed in the other groups.

Batch 2 of B group did not show any contamination with *S. aureus*, *E. coli* and *Shigella* spp. However, an average of ~ 40 cfu/L of *Klebsiella* app was found in the samples of batch 2. On the other hand, all the samples of the batch 1 were spoiled with pathogens where the cfu average was as ~ 13.0 (*S. aureus*), ~ 17.0 (*E. coli*), 37.0 (*Klebsiella* spp), and ~ 23.0 cfu/mL (*Shigella* spp) (Table 6). Thus, high potential hygienic practices are required from batch 1 farm (Table 6).

Dairy farm of the batch 2 (D group) was found to be the most hygienic and animal health management committed as none of this work candidate pathogens were detected from. In contrast, all the samples of the batch 1 was found to be contaminated with pathogens. Milk bacterial-dirtiness cfu averages were as ~ 28.0 (*S. aureus*), 1.5 (*E. coli*), ~ 12.0 (*Klebsiella* spp), and 16.0 cells/mL (*Shigella* spp) (Table 6). Animal health regulations and farm hygienic conditions highly need a revision.

As for K group, batch 1, it was noticed to be free of *S aureus*, *E. coli* and *Shigella*, but it was highly contaminated with *Klebsiella* spp producing a cfu average of ~ 100.0 including all samples. On the other side, all samples of batch 2 were spoiled with *S. aureus* (~29.0 cfu/mL), only 1sample with *E. coli* (~ 7.0 cfu/mL), and only 2 samples with *Klebsiella* spp (~3 cfu/mL) whereas no *Shigella* spp were found in any sample (Table 6).

Table 6: Prevalence and percentages of *S aureus, E. coli, Shigella* spp., and *Klebsiella* spp. in this work raw-milk samples.

Pathogens	**Z group**		**B group**		**D group**		**K group**	
No. of + Samples	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2
S. aureus (+) samples	NG	NG	13.3 cfu* (10/10)	NG	28.4 cfu* (10/10)	NG	NG	28.7 cfu* (10/10)
E. coli (+) samples	NG	NG	16.9 cfu* (8/10)	NG	1.5 cfu* (10/10)	NG	NG	7.4 cfu* (1/10)
Klebsiella spp (+) samples	99 cfu* (8/10)	NG	37.0 cfu* (6/10)	40.1 cfu* (10/10)	12.5 cfu* (10/10)	NG	96.9 cfu* (10/10)	12.9 cfu* (2/10)
Shigella spp (+) samples	80 cfu* (10/10)	57 cfu* (10/10)	23.3 cfu* (9/10)	NG	15.7 cfu* (10/10)	NG	NG	NG

NG = No Growth, * = cfu mean of the positive sample

It was suggested that the main potential *S. aureus* risk factors prevalence are the lack of bactericidal teat dipping before and after milking and tick infestation (Gebremedhin *et al*., 2022), personnel and no individual tools used for each sheep udder cleaning (Borena *et al*., 2023). The difference in *Staphylococcus* occurrence between raw-milk and milk-derived products as found in this study (30/80) is based on the milk storage, handling, use of unhygienic utensils, and milking circumstances (El-Malt *et al*., 2013; Lee *et al*., 2012).

Most common *E. coli* contamination occurred on the base of a cross faecal-udders transmission (Ghali-Mohammed *et al*., 2023). It was found that Shiga-toxin-producing *E. coli* (STEC) is the most common strain that has been detected in raw milk while enterotoxigenic *E. coli* (ETEC), and enteropathogenic *E. coli* (EPEC) were also found in raw milk but in much lower rates. However, due to short of chemical and facilities, *E. coli* strains have not been evaluated. Nevertheless, the results arrived at in the current study demonstrate that *Klebsiella* is more common in the raw milk samples compared to *S. aureus* and *E. coli*.

Prevalence of *Klebsiella* spp throughout the dairy farms environments is predictable due to their presence in animal feces (Munoz *et al.*, 2007). Control of *Klebsiella* mastitis and fecal-udder contamination are the crucial procedures to decrease the *Klebsiella* presence in raw milk (Zadoks *et al*., 2011). Therefore, restricting the *Klebsiella*-mediated mastitis infection and decreasing the fecal contamination are recommended for the reducing of *Klebsiells* vegetative cells in raw milk. Several suggestions have been proposed to obtain healthier raw milk with lower number of *Klebsiella* such as more attention should be paid to bedding hygiene and bedding replacement, alley hygiene and maintenance of alley scrapers (Munoz *et al*., 2008). In summary, *Klebsiella* spp prevalence is highly related to manure and that keeping the bedding place in hygienic condition is not enough to prevent exposure of adder to potential mastitis pathogens (Zadoks *et al*., 2011).

Some species of *Shigella* are responsible for shigellosis; however, determination of this milk-born pathogen species was not an aim of the current study. In spite of the predominance of *S. dysenteriae* (serovar A) in Africa (Elkenany *et al*., 2022), *S. flexneri* has been mentioned as the main causative agent of shigellosis in the third world (Bintsis, 2017).

A high rate of *Shigella* prevalence (39/80) in this study could be due to neglecting in hygienic standards during milk processes (Ahmad and Shimamoto, 2014; Hale and Keusch, 1996). Furthermore, water and faeces were also found to be important sources of the *Shigella* propagation (Litwin *et al*., 1991) in addition to the mode of milking where mechanical milking is more likely to produce *Shigella*-mediated contamination than the manual milking (Oueslati *et al*., 2011).

Due to the absence of some specific chemicals, equipment, facilities, and time shortage, Mesophilic raw milk-associated bacteria like *Listeria monocytogenes*, *Brucella* spp, and *Campylobacter* spp. along with psychrophilic bacteria, like *Pseudomonas* spp., was not addressed in any analysis. Identification of isolates was carried out by applying some traditional biochemical reactions but not via isolation and sequencing the 16S rRNA genes techniques which are more reliable. Species of *Shigella* and *Klebsiella* in addition to the sub-species of *S. aureus* and *E. coli* were not determined. Moreover, somatic cells were not estimated due to the lack of time and facilities.

CONCLUSION AND RECOMMENDATIONS

The results of this study revealed that raw milk is contaminated by total bacteria count and at least four potential pathogens; *S aureus*, *E. coli*, *Shigella* spp., and *Klebsiella* spp. Batch 2 (D group) found to be healthier milk collection as none these addressed pathogens were found in any sample. No *S. aureus* or *E. coli* was found in any sample of Z group (batch 1 and 2), B group (batch 2), D group (batch 2), and K group (batch 1). Batch 1 of the B and D groups were the most contaminated milk as the four subjected pathogens were found in their samples. The detected bacteria from collected raw milk were *Staphylococcus aureus* 37.5% (30/80), *Escherichia coli* 23.75% (*n*=19), *Klebsiella* spp 57.5% (*n*=46), and *Shigella* spp 48.75% (*n*=39). Different significant factors were associated with raw milk contamination such as employee hand washing, unclean milk containers, milking process, and animal disease. Depending on the current study finding, the following recommendations are proposed: farmers' general hygiene and cleaning containers should be committed. Untreated milk with and sanitary practice during collecting and transporting milk, particularly in the summer season is recommended. Local and national government must establish a diagnostic center to test the raw milk bacteriologicaly prior marketing. Farmers should be provided with easy access to the veterinary clinics. Finally, no *Salmonella* species were detected in any raw milk sample.

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