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Survey of Bacterial Contamination in Raw Cow's Milk from Different Farms in Benghazi City – Libya

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ABSTRACT

Samples of raw cow's milk collected randomly from 10 individual farmers from different regions around Benghazi including Sidi Khalifa (SK), Al-Kuwaifieh (KU), Sidi Mansour (SM), Deriana (DE), Al-Qawarsha (QA), Al-Hawari (HA), Sidi Faraj (SF), Bouatni (BO), Al-Fayd (FA) and Al-Nwaqia (NW). The total count of bacteria, coliform bacteria count, *Staphylococcus* count, the probability presence of *Salmonella* in milk samples were determined. The results showed that most samples were highly contaminated as the total count ranged from 5.74 (Nwaqia) to 8.44 (Sidi Khalifa) $\log_{10}$CFU/ml. Coliform bacteria were detected in all samples and the numbers exceeded the permissible limit in the raw milk. No *Salmonella* was detected in all tested samples. 58 isolates obtained from milk samples were identified as following; *E. coli* (34%), *Enterobacter* spp. (10%), *Citrobacter* spp. (9%), *Klebsiella* spp. (9%), *Proteus* spp.(5%), *Pseudomonas* spp.(7%), *Bacillus subtilis* (19%), and *Streptococcus mutans* (7%). The failure to follow good hygiene practices in raw milk production by individual farmers resulted in a high bacterial contamination in milk which exposes the consumer to contracting milk-borne diseases.

INTRODUCTION:

Milk and milk products have been a significant part of human food since ancient times, and have a protuberant role in the nutrition(1). There are many nutrients in milk and dairy products, e.g. protein, vitamins, calcium, phosphorus, magnesium, zinc, etc., which are essential for the healthy life of human(2). Researchers stated that the average components of cow's milk are 87% water, 4-5% lactose, 3% protein, 3-4% fat, 0.8% mineral salts, and 0.1% vitamins (3)(4).

Milk production in the world has increased recently. According to the FAO, worldwide milk production get hold of 852 million tons in 2019, which was a rise of 1.4 % from 2018(5). In same context, in Africa, milk production in 2019 is estimated at 46.8 million tons, an increase by 0.3 % from 2018(5). In 2013, the milk consumption in Libya was reported to be ~75.0 kg/capita/years(6).

It is difficult to produce milk and milk products completely free from microbial contamination. Therefore avoiding high microbial load during production process is the main consideration for safe dairy products(7). Several factors contribute in raw milk contamination such as improper milking methods, workers, soiled hands, improperly washed and disinfected machinery (including bulk milk tanks), failure to detect irregular milk (mastitis bacteria, blood, and clots); foreign bodies, particularly in milking machinery and bulk tanks (8)(9).

Fresh and processed milk are a good media that promote the growth of many microorganisms and therefore it is known as efficient vehicle for transmission of disease to consumers (10) . The disease-causing agents in milk are; *Salmonella* spp., *Mycobacterium bovis, Corynebacterium* spp., *Clostridium perfringens, Yersinia enterocolitica, Coxiella burnetii, Brucella, Staphylococcus, Campylobacter jejuni, Mycobacterium avium, Listeria* spp., *Escherichia coli,* and coliform bacteria (11).

Many studies illustrated contamination of raw milk with bacteria. In Ghana, Donkor, *et al.* (12) isolated several species of bacteria from raw milk, such as *Yersinia, Klebsiella, Proteus, Enterobacter, Escherichia coli, Staphylococcus, Bacillus* and *Mycobacterium* spp. Garedew, *et al.,* (10) reported that coliform bacteria, including *Escherichia coli, Klebsiella pneumoniaee, Alcaligenes feacalis,* and *Enterobacter earogenes,* were detected in milk samples obtained directly from cows at a frequency of 47.1%, 26.5%, 14.7%, and 11.8%, respectively. Foodborne

diseases related to the ingestion of milk products have been predominantly associated with *Campylobacter jejuni, Escherichia coli* O157*:* H7, *Listeria monocytogenes,* and *Salmonella entertica* in the past few decades (13).

There is a significant responsibility on dairy producers, retailers and manufacturers to produce high quality and safe milk and milk products. In eastern part of Libya, there are some private farms distributed around Bengazi city (The second largest city after the capital, Tripoli) that raising cow in small herds (consist of 3 to 20). Cows are usually fed hay, straw, dry bread, and barley, while the concentrated feed is introduced in short supply. It is noticed that during milking process good hygiene practices are not followed which may lead to an increase the threat of public health with foodborne diseases. Also, contaminated raw milk will deteriorate faster during storage and transportation(14). Therefore the goal of this study is to determine the level of bacterial contamination in cow's milk and the degree of the application of hygiene measures during manually milking in some farms in Benghazi city.

MATERIALS AND METHODS:

Samples collection:

Samples of cow's milk were obtained from 10 individual farmers from different regions around Benghazi (Sidi Khalifa (SK), Al-Kuwaifieh (KU), Sidi Mansour (SM), Deriana (DE), Al-Qawarsha (QA), Al-Hawari (HA), Sidi Faraj (SF), Bouatni (BO), Al-Fayd (FA), and Al-Nwaqia (NW)). The samples were collected in sterile glass bottles and transported directly (into the icebox) to the Laboratory of microbiology in the faculty of Agricultural /Benghazi University. Each sample was given an individual number and examined as a separate unit.

Preparation of sample

10 ml of each milk sample was transferred to a sterile blending container, previously sterilized by washing with hot water and rinsing with ethanol alcohol (95%) and then allowing the remaining alcohol to burn. The sample was blended with 90 ml of sterile peptone water (0.1%), in a bottle with good shaken for 2 min to obtain a homogenate mixture with 1/10 dilution(15). The homogenate mixture was transferred to a sterile 500 ml bottle, mixed well by swirling the bottle then the bottle with loosening cap incubated at 37°C for 24 hr to isolate *salmonella*(16).

Determination of total bacterial count Plate Count Agar (PCA)

1ml of homogeneous mixture sample was transferred to a tube containing 9 ml of peptone water to obtain a concentration of 0.01 (1/100). A serial dilution was conducted using peptone water to research a final dilution of 10^{-7}. 1 ml of each dilution transferred into three sterilized Petri dishes, then about 10 to 15 ml of plate count agar medium tempered to 45°C were poured into plates. The contents of the plates were mixed thoroughly incubated at 37°C for 24 - 48 hr.(17). After incubation, the number of colonies (CFU/ml) was counted using a standard plate with 25 - 250 colonies(18). To prove the total number of bacteria, the number of poured plates containing 30 to 300 colonies was used to estimate the total number of bacteria in milk samples. The total bacteria counts were calculated by multiplying the inverse dilution factor by the average number of colonies in the plates (three replicates per dilution). The colony formation unit /ml (CFUml) of the sample was converted into $\log_{10}$CFU/ml.

Total Coliform Count Test (TCC)

TCC was tested using the method described by Al-Karablieh, et al. (16). 1 ml of each dilution was transferred into three sterilized Petri dishes. About 10 to 15 ml of Violet Red Bile (VRB) agar, tempered to 45 °C, were poured into plates, the contents of the plates were mixed and left to solidify. Duplicate plates and agar control plates were run for each series of samples. The plates were inverted and incubated at 35°C for 24 hours. After incubation, the number of coliform colonies (CFU/ml) was counted, using plates with 15 - 150 coliform colonies(19). Coliforms in VRBA appear as typical dark red colonies normally measuring at least 0.5 mm in diameter on an uncrowded plate(19).

Identification of Coliform Bacteria

Coliform bacteria were identified by the total number of characteristic colonies. Coliform bacteria form dark red to purple colored colonies larger than 0.5mm in diameter, usually surrounded by a purple zone but sometimes without. These are considered as typical colonies of coliforms and no further confirmation is required(20). Typical colonies were confirmed by Gram's staining followed by different biochemical tests (motility test, catalase test, oxidase test, citrate utilization, indole test, MRVP test, and Triple Sugar Iron (TSI) test).(18)(19)(20). 58 different isolates were picked and identified by Gram's staining, microscopic examination for

morphological characters, cultural and biochemical tests. All media were obtained from Oxoid, Basingstoke, England).

Isolation and Identification of *Staphylococcus aureus*

0.1 ml of both dilutions 10^{-3} and 10^{-4} were spread on the surface of plates containing gelatin mannitol salt agar (Oxoid, England) (two replicates per dilution) medium using L-shaped glass rod. The plates were then incubated at 35°C for 24 hours. *Staphylococcus* bacteria appear on medium as yellow color colonies (21)(22).

Isolation and Identification of *Salmonella*

Salmonella test was conducted as described by Al-Karablieh *et,al*. (16). To promote *Salmonella* growth the remaining of 1/10 dilution of the sample is taken with the nutrient broth, placed in a sterile flask and incubated at 37°C for 24 hours. After incubation, a full needle of the preparation was aseptically struck on *Salmomella Shigella* Agar (S.S. Agar) and Xylose Lysine Deoxycholate (XLD agar media) and incubated at 37°C for 24 hrs. At the end of the incubation period, the dishes were tested for the presence of *Salmonella*, where their colonies appear on the XLD media in the form of pink-red in color with or without a black center, while on S.S Agar it appears as colorless colonies with or without a black center. Typical colonies were confirmed by staining followed by different biochemical tests(23).

Statistical analysis

Descriptive statistics was used to collect data and colony-forming units/g (CFU/g) were counted and converted to Log_{10}CFU/g. The data were analyzed with SPSS software (Statistical Package for Social Science version 23, IBM/SPSS). ANOVA was performed and Duncan's test was used as a post hoc test. Mean differences were considered significant at values of $P < 0.05$.

RESULTS AND DISCUSSION:

The average of Total Bacterial Count (TBC) of milk samples in the ten regions in Bengazi city are illustrated in Table (1). Results indicated that there were significant differences ($P<0.05$) between all tested regions. Sidi Khalifa had significantly the highest TBC (8.44 log_{10}CFU/ml) whereas Al-Nwaqia had the lowest one (5.74 log_{10}CFU/ml).

Table No. 1: Average number of TBC (log_{10}CFU/ml) of milk in different tested regions

*Region	Mean	SD	Minimum	Maximum
SK	8.44^{a}	0.02	8.41	8.46
KU	5.75^{f}	0.07	5.67	5.81
SM	8.19^{b}	0.07	8.11	8.26
DE	7.11^{e}	0.03	7.07	7.13
QA	6.88^{e}	0.09	6.80	6.98
HA	7.80^{c}	0.02	7.78	7.81
SF	7.60^{cd}	0.03	7.59	7.63
BO	6.97^{e}	0.01	6.95	6.98
FA	7.56^{d}	0.01	7.59	7.61
NW	5.74^{f}	0.08	5.66	5.81

*= Sidi Khalifa (SK), Al-Kuwaifieh (KU), Sidi Mansour (SM), Deriana (DE), Al-Qawarsha (QA), Al-Hawari (HA), Sidi Faraj (SF), Bouatni (BO), Al-Fayd (FA), and Al-Nwaqia (NW) In colum, means with the different letters are significantly different at p <0

The findings displayed that coliform bacteria were present in all milk samples (Table 2). Coliform bacterial count varied significantly (p<0.05) between milk samples from all regions and the highest TCC (6.70 log_{10}CFU/ml) was detected in samples of Al-fayd region whereas the lowest count (2.03 log_{10}CFU/ml) was obtained from Bouatni milk samples. All milk samples exceeded the recommended limit set for this group of bacteria.

Table No. 2: Average number of TCC (log_{10}CFU/ml) of milk collected from tested regions

*Region	Mean	SD	Minimum	Maximum
SK	5.08^{b}	0.07	5.00	5.14
KU	5.58^{b}	0.05	5.53	5.62
SM	3.82^{cd}	0.32	3.58	4.18
DE	4.40^{c}	0.09	4.30	4.48
QA	3.61^{d}	0.06	3.54	3.65
HA	2.48^{e}	0.08	2.40	2.54
SF	3.30^{d}	0.05	3.26	3.35
BO	2.03^{e}	0.05	2.00	2.08
FA	6.70^{a}	0.04	6.66	6.73
NW	5.40^{b}	0.28	5.08	5.60

*= Sidi Khalifa (SK), Al-Kuwaifieh (KU), Sidi Mansour (SM), Deriana (DE), Al-Qawarsha (QA), Al-Hawari (HA), Sidi Faraj (SF), Bouatni (BO), Al-Fayd (FA), and Al-Nwaqia (NW) In colum, mean with different letters are significantly different at $p<0.05$

Table No. 3: Average number of total staphylococcus count (TSC) (log_{10}CFU/ml) of milk collected from tested regions

*County	Mean	SD	Minimum	Maximum
SK	6.41^{b}	0.04	6.36	6.43
KU	5.62^{c}	0.07	5.57	5.70
SM	6.13^{b}	0.11	6.00	6.20
DE	3.10^{d}	0.09	3.00	3.18
QA	7.09^{a}	0.02	7.08	7.11
HA	2.49^{e}	0.08	2.40	2.56
SF	2.00^{f}	0.001	2.00	2.00
BO	1.70^{f}	0.01	1.69	1.71
FA	2.60^{e}	0.001	2.60	2.60
NW	2.69^{e}	0.01	2.68	2.70

*= Sidi Khalifa (SK), Al-Kuwaifieh (KU), Sidi Mansour (SM), Deriana (DE), Al-Qawarsha (QA), Al-Hawari (HA), Sidi Faraj (SF), Bouatni (BO), Al-Fayd (FA), and Al-Nwaqia (NW)

In column, means with different letters are significantly different at $p<0.05$.,

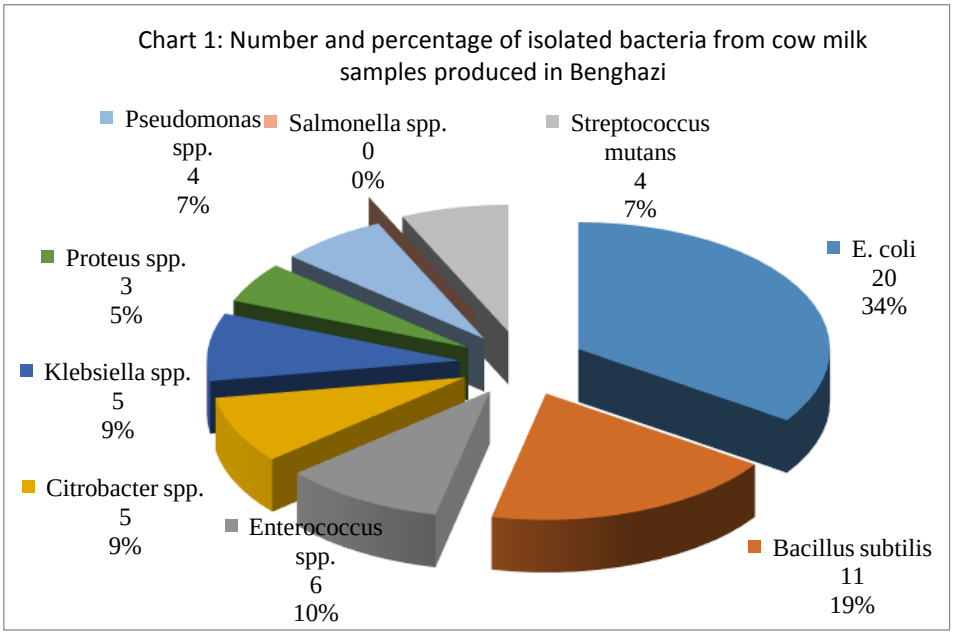
Table (3) presents that QA recorded the highest count (7.09 log_{10}CFU/ml) of *Staphylococcus* bacteria (TSC) followed by SK and SM (6.41and 6.13 log_{10}CFU/ml respectively). *Staphylococcus* counts were significantly the lowest in BO and FA (1.70 and 2.00 log_{10}CFU/ml respectively).

Table (4) illustrates the results of different biochemical reactions used in the definition of coliform species in milk samples. The identified species were *E. coli*, *Enterobacter* spp., *Citrobacter* spp., *Klebsiella* spp. The number and percentage of bacterial isolates (58 isolates) of milk samples produced in Benghazi (Chart 1) was *E. coli* as prevalent species (20, 34%) , *Enterobacter spp*. (6,10%), *Citrobacter spp*. (5, 9%), *Klebsiella spp*. (5, 9%), *Proteus spp*.(3, 5%), *Pseudomonas spp*.(4, 6.90%), *Salmonella spp*. (0, 0%), *Bacillus subtilis* (11, 19%), and *Streptococcus mutans* (4, 6.90%).

Table No. 4: Biochemical reactions for identification of bacterial isolates from milk samples produced by different regions in Benghazi city

Isolated bacteria	TSI				SIM			Simmon	VP	MR	Catalase	Gram Stain
	Slant	Bottom	H_2S	Gas	Sulfide	Indole	Motility					
E. coli	A	A	-	d	-	+	+	-	-	+	+	-
Enterobacter	Ak/A	A	d	d	d	d	+	+	-	+	+	-
Citrobacter	A	A	-	-	-	+	+	-	-	+	+	-
Klebsiella	A	A	-	+	-	d	-	+	+	-	+	-
Proteus	Ak	A	+	+	+	-	+	-	-	+	+	-
Pseudomonas	Ak	Ak	-	-	-	-	+	+	-	-	+	-
Salmonella	Ak	A	V	V	V	-	+	V	-	+	+	-
Bacillus	d	d	-	-	-	-	+	+	+	-	+	+
Streptococcus	A	A	-	-	-	-	-	+	+	+	-	+

Alk: alkaline reaction, A: Acid reaction, MR: Methyl Red, VP: Vogas Proskauer, +: positive result, - : Negative result, d: different results, V: Variable



The obtained bacterial contents were compared with that of the European specifications for milk products, which stipulated that the total number of bacteria permitted in raw milk intended for direct use of humans should not exceed $5x10^4$ CFU/ml. The European specifications confirmed that raw milk intended for industrial processes should not exceed 10^5 CFU/ml, and the number of *Staphylococcus* bacteria should not exceed from 100 to 500 cells/ml (24). The total number of bacteria exceeded the permissible limits in the total number of bacteria, as well as the number of coliform bacteria and the number of *Staphylococcus aureus* bacteria (Table 1). Our results showed that the most common coliform bacteria in raw milk samples were *E. coli*, and *Enterococcus* spp. *Klebsiella* spp, *Protus* and *Citribacter* spp. The total coliforms, *E. coli* and other enteric bacteria are reliable indicators of fecal pollution in poor sanitary conditions of milk, water, food and other dairy products. In this study recovery of *E. coli* from milk samples is an indicative of possible existence of enteropathogenic and/or toxigenic microorganisms which could constitute a public health hazard. In the present study other species of bacteria were detected including *Bacillus subtilis* and *Pseudomonas* spp. (Table 2, chart 1). These results indicated that the milk might be contaminated during milking, handling, transport, and manual packing, because the hygiene factors were not taken into consideration. Our findings are consistent with several previous studies that have shown the presence of coliform bacteria (25), *Staphylococcus aureus*(26), *Escherichia coli*(27), as well as, *Bacillus spp*, *Clostridium spp* and *Yersinia enterocolitica* (27)(28) and *Klebsiella pneumoniae* (10) in raw milk samples. In

conclusion, results showed that all tested bacteria exceeded the permissible limits recommended by European specifications. This rises the amount of overall allowed bacteria in raw milk suggests that farmers in the research region do not stick with good manufacturing practices to meet safety requirements.

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