Project Report

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SIR P. T. SCIENCE COLLEGE

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SIR P.T.SCIENCE COLLEGE, MODASA

(Managed by THE M. L. GANDHI HIGHER EDUCATION SOCIETY) [UGC 2F, 12B RECOGNISED]- [NAAC- ACREDITED B^{++}]

CERTIFICATE

This is to certify that the project work on

ISOLATION OF MILK FLORA FORM RAW MILK

entitled is carried out by students mentioned below. The Project work allotted them first year Bachelor of Science during the academic year 2022-2023.

The project has been approved as it satisfies the academic requirements in respect of project work prescribed for the first year Bachelor of Science NEP 2020.

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INTRODUCTION

Milk is a highly nutritious food that can be obtained from a variety of animal sources such as cows, goats, sheep and buffalo, as well as humans, for human consumption. However, the high nutrient content of these milks, which includes proteins, fats, carbohydrates, vitamins, minerals and essential amino acids (Supporting information, Table S1), all at a near neutral pH and at a high water activity, provides an ideal environment for the growth of many microorganisms. Some of these nutrients are directly available to all microorganisms, while others are provided following the metabolism of major components by specific populations to release components and metabolites that are used by others (Frank, 1997). It is generally accepted that the lactic acid bacteria (LAB), a group of bacteria that ferment lactose to lactate, are a dominant population in bovine, goat, sheep and buffalo milk, prior to pasteurization. The most common LAB genera in milk include *Lactococcus, Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Enterococcus.* Psychrotrophic populations, which particularly establish themselves during cold storage, are also a major component and frequently include *Pseudomonas* and *Acinetobacter spp*. Other strains of non-LAB genera are also encountered in milk, as well as various yeasts and moulds (Quigley *et al*., 2011). Human milk on the other hand is typically dominated by Streptococcus, Staphylococcus, Lactobacillus and *Bifidobacterium spp*. (Martın *et al*., 2007). The specific composition of the milk micro biota directly impacts on the subsequent development of dairy products. Microorganisms can bring about the fermentation of milk through the production of lactate and have a variety of different impacts on the sensory, texture, flavour and organoleptic properties of resultant products (Wouters *et al*., 2002). Microorganisms can also negatively impact on milk quality and shelf life; for example, psychrotolerant bacteria can proliferate during refrigeration and, through the production of extracellular lipases and proteases, result in spoilage (Desmasures & Gueguen, 1997; Hantsis-Zacharov & Halpern, 2007). The microbial composition of milk can also have health related implications in that the consumption of raw milk contaminated with pathogens can lead to, in some cases, severe illness (Oliver *et al*., 2009). In contrast, it is claimed that other raw milk microorganisms.

Methods employed to determine the microbial composition of milk many microbial communities are complex; that is, they are comprised of many different taxonomical groups of microorganisms. Raw milk is an example of an environment that contains a diverse and complex microbial population (Quigley *et al*., 2011; Vacheyrou *et al.*, 2011).

Most of our knowledge with respect to the identity of the microorganisms that are present in raw milk, and resultant dairy products, has been gained through the growth or 'culturing', and subsequent analysis, of these microorganisms. The ultimate identification of these cultured microorganisms involves phenotypic and/or genotypic methods. Phenotypic methods are those which have been traditionally employed and involve the growth of microorganisms in microbiological media (either general or selec tive) supplemented with morphological, biochemical or physiological characterization (Quigley *et al*., 2011). These testing methods are still the standard in industrial settings and typically involve tests to determine total bacteria counts, reflecting general milk quality, or to detect specific pathogens or other microorganisms, which indicate whether contamination has occurred. Populations frequently tested for include thermoduric populations (resisting pasteurization), sulphate-reducing *clostridia*, *Listeria monocytogenes*, *Salmonella*, coagulase-positive *staphylococci*, *Escherichia coli*, *Enterobacteriaceae*, *coliforms* and *Bacillus cereus* among others. These tests generally rely heavily on the use of microbiological broths or agars that selectively support the growth of the target microbial population and often include further confirmatory biochemical analysis. These approaches are usually low-tech and inexpensive but are relatively labour intensive and time-consuming, and in some cases, insufficient discriminatory power can be a problem. More recently, considerable efforts have been made to develop more rapid, high-throughput tests.

Sources of milk microorganisms

Milk in healthy udder cells is thought to be sterile (Tolle, 1980) but thereafter becomes colonized by microorganisms from a variety of sources, including the teat apex, milking equipment, air, water, feed, grass, soil and other environments. The bovine teat surface can contain a high diversity of bacteria (Braem et al., 2012; Monsallier *et al*., 2012; Verdier-Metz *et al*., 2012). In one particularly detailed study, culture-dependent methods revealed that the bacteria present could be classified at the phylum level as *Firmicutes* (76%), *Actinobacteria* (4.9%), *Proteobacteria* (17.8%) and *Bacteroides* (1.3%). When this approach was supplemented by a clone library sequencing–based approach, some additional phyla, that is, *Planctomycetes*, *Verrucomicrobia*, *Cyanobacteria*, *Chloroflexi* and unclassified Bacteria, were detected at low levels (Verdier-Metz *et al*., 2012). Notably, a large percentage of the reads from this and other studies (Fricker *et al*., 2011) corresponded to as yet unidentified bacteria. Of those which could be identified, many

corresponded to technologically impor tant bacteria such as Lactobacillus, Leuconostoc and Entero coccus spp. Bacteria that can be involved in flavour, aroma and colour development in cheese such as coagulase-nega tive staphylococci as well as Arthrobacter, Brevibacterium and Corynebacterium spp. were also detected. However, some of the microorganisms detected on the teat surface, for example, *Solobacterium, Clavibacter* and *Arcanobacterium spp*., have not been identified in milk (Verdier-Metz *et al*., 2012), presumably reflecting a lack of competitiveness in milk environments should transfer occur. It was also noted that the composition of the microbial community on the teat surface varied qualitatively and quantitatively from one farm to another (Verdier-Metz *et al*., 2012). This can be attributed to many different factors; for example, microorganisms associated with bedding material can contaminate the surface of teat and thus potentially enter milk (Vacheyrou *et al*., 2011). Similarly, milking machines can contain a reservoir of microorganisms, and thus, unsurprisingly, differences between machines and related practices can influence the microbial population of the milk collected (Michel *et al*., 2006). With respect to more general environmental factors, it has been observed that the microorganisms present in cows' milk depend on whether animals are fed indoors or outdoors, with an increase in Staphylococcus spp. during outdoor feeding (Hagi *et al*., 2010), on the location of the animals (Bonizzi *et al*., 2009) and on the lactation stage (Callon *et al*., 2007). An intense study was carried out to relate the microorgan isms detected in milk to where they can be found on the farm (Vacheyrou *et al*., 2011). These results highlighted 141 bacterial species, representing 54 genera, from throughout the farm. There were 25 genera detected in these milk samples, and many of these, including *Aerococcus, Streptococcus, Propionibacterium, Acinetobacter, Bacillus, Ochrobactrum, Pseudomonas, Psychrobacter, Staphylococcus, Sphingomonas, Enterobacter, Pantoea, Brachybacterium, Corynebacterium, Kocuria, Microbacterium* and *Pseudoclavibacter*, were also detected in different areas throughout the farm including teat surfaces, milking parlours, hay, air and dust. Also present in milk, but not detected in the farm environment, were technologically relevant bacteria such as *Lactococcus, Lactobacillus* and *Enterococcus* as well as *Leucobacter, Deinococcus* and *Paracoccus.* Similarly, a large number of other taxa were detected in the farm environment, but not in milk (Vacheyrou *et al*., 2011). Finally, it is notable that the implementation of strict hygiene standards brings about a reduction in the microbial load of milk, including a reduction in populations of technological importance, which can, in turn, impact negatively on cheese manufactured using traditional or artisanal approaches (Monsallier *et al*., 2012). Indeed,

Mallet *et al*. (2012) recently reported a one-magnitude reduction in the levels of technologically relevant *lactococci* present in raw milk relative to what had been detected 15 years before in raw milk collected from the same area (Desmasures & Gueguen, 1997). These populations seem to be particularly sensitive to the evolution of farm practices, as other populations, such as Pseudomonas, Lactobacillus and yeast populations, did not differ across the two studies. While it is important to ensure that the quality of milk is maintained at high levels, producers of traditionally manufactured raw milk cheese should be aware that certain farming practices may negatively impact on distinctive flavours and aromas as a consequence of limiting the numbers of specific micro organisms and may need to compensate through the introduction of starters and adjunct strains.

The microbial composition of different milk types

Although the largest proportion of commercially produced milk worldwide comes from cows, there are a number of other animal sources of milk that is used for human consumption. These include quite common sources such as goats, sheep, buffalo and others utilized in more specific regions such as camel milk in African and Arab countries and yak milk in Asian countries. This section will review recent findings on the microbial con tent of these various milks. We will also discuss an issue that has been receiving ever more attention in recent years; that is, the microbial composition of the human milk that is consumed by infants only (Box 1). Cows' milk Cows' milk is produced on a massive scale. In 2012, the EU produced c. 139 million tones of cows' milk followed by the United States with 90 million tonnes (http://www. dairyco.org.uk/market-information/supply-production/ milk-production/world-milk-production/). This milk is employed in many ways, including direct consumption and the manufacture of dairy products and milk powders. Raw cows' milk has the potential to contain a diverse bacterial population as highlighted previously (Quigley *et al*., 2011).Typically, cows' milk contains a significant LAB population that includes *Lactococcus* (8.2 9 101–1.4 9 104 CFU mL 1), *Streptococcus* (1.41 9 101–1.5 9 104 CFU mL 1), *Lactobacillus* (1.0 9 102 3.2 9 104 CFU mL 1), *Leuconostoc* (9.8 9 101–2.5 9 103 CFU mL 1) and *Enterococcus spp*. (2.57 9 101–1.58 9 103 CFU mL 1;). A number of other microorganisms can be present in significant proportions. These include psychrotrophs, such as *Pseudomonas, Acinetobacter and Aeromonas spp*., which flourish during cold storage (Raats *et al*., 2011). However, while the bacterial composition of cows' milk has been extensively studied for quite some time, new developments with respect to DNA

sequencing technologies have highlighted that the diversity of these bacteria is greater than that originally appreciated (Table 1). Indeed, a recent study applied high-throughput DNA sequencing to examine the bacterial population of raw cows' milk that was to be used for cheese production (Masoud *et al*., 2012); 256 bacterial species were detected, of which Streptococcus thermophilus and Lactococcus lactis dominated in the milk, representing 43.7% and 19% of reads, respectively. A number of other microorganisms that had previously been associated with raw milk, including *Acinetobacter, Aeromonas, Brevibacterium, Corynebacterium, Lactobacillus, Pseudoalteromonas, Pseudomonas and Staphy lococcus*, which represented between 1.3% and 3.7% of the total reads, were also detected. A large subpopulation of taxa, which each corresponded to a detailed insight into the bacterial composition of milk, and it is likely that these technologies will be used increasingly in future to investigate the factors that influence the composition of cows' milk. Goats' milk Goats' milk production represents about 2.1% of global milk production (Tsakalidou & Odos, 2012). It is an important commodity that has gained increased interest as an alternative to cows' milk, due to evidence that it is less likely to induce allergies (Park, 1994). Goats' milk also differs from cows' and sheep's milk by virtue of having greater levels of iron bioavailability (Boyazoglu & Morand-Fehr, 2001) as well as containing smaller fat globules, having a higher content of fatty acids and forming a softer curd during subsequent fermentations, in turn leading to greater digestibility (Klinger & Rosenthal, 1997). Goats' milk is most frequently used for cheese making, usually at farm level or in small dairies. Goats' milk cheeses are particularly common in Mediterranean countries and south-east Europe (Pirisi *et al*., 2007). Goats' milk is also typically dominated by LAB, including species of *Lactococcus (*3.7 9 106 CFU mL 1), *Lactobacillus* (1.34 9 105 CFU mL 1), *Leuconostoc* (3.27 9 103 CFU mL 1) and *Enterococcus (*2.95 9 102 CFU mL 1), as well as *Enterobacteriaceae*, *Micrococcaceae*, moulds (filamentous fungi) and yeasts (Alonso-Calleja *et al*., 2002; Tamagnini *et al*., 2006; Nikolic *et al*., 2008). Callon *et al*. (2007) relied on the use of selective microbiological media, SSCP analysis as well as restriction fragment length polymorphism (RFLP) typing of isolates to exam ine the microbial diversity of 118 goats' milk samples taken from one herd throughout one lactation year to reveal the presence of a diverse bacterial population in the milk In addition to microorganisms commonly encountered in milk, such as those listed above, some species were identified that are not typically associated with goats' milk or that had previously only been associated with cheeses, including a number of *corynebacteria* and *brachybacteria*. Another unexpected

finding was the pre ence of several halophilic species not previously associated with milk, including *Jeotgalicoccus* psychrophilus, *Salinicoccus sp.,* Dietzia maris, *Exiguobacterium, Ornithinicoccus sp. and Hahella chejuensis*. The significance of the presence of these microorganisms with respect to health, safety or product development is not known. Through this approach, it was also revealed that milks collected during winter were dominated by the presence of *Lactococcus* and *Pseudomonas,* those from summer by *Pantoea agglomerans* and *Klebsiella* and those from autumn by *Chryseobacterium indologenes, Acinetobacter baumannii, Staphylococcus, Corynebacteria* and yeasts. While these variations can be attributed to differences in feed, the authors suggested that other factors, such as weather conditions and the health of the animal, were also important (Callon *et al*., 2007). There has not been an in-depth assessment of the microbiology of goats' milk since this study, perhaps next generation sequencing technologies could have the potential to be very revealing.

Sheep milk Milk is rarely consumed but still constitutes. 1.3% of global milk production as it is often employed throughout Europe in the development of cheese (Tsaka lidou & Odos, 2012). Sheep milk is dominated by LAB, with mesophilic bacteria representing 102–106 CFU mL 1, while psychrotrophic populations correspond to 102 104 CFU mL 1 (Fotou *et al*., 2011). Studies assessing the impact of storing sheep milk at refrigeration temperature highlighted increases in psychrophiles, but also in mesophiles. Unsurprisingly, the thermoduric population did not increase. These general trends are also affected by temperature and the length of storage (de Garnica *et al*., 2011). Other bacteria that have been detected on occasion can include microorganisms of concern from a milk safety perspective including E. coli, Salmonella, *Staphylococcus aureus, Bacillus and Clostridium perfringens* (Fotou *et al*., 2011). The location can affect both the nutritional composition and microbial composition of sheep milk. A correlation has been noted between milks with a higher fat content and greater counts of LAB, coliforms and moulds. In populations of *streptococci and S. aureus,* there was an increase and a decrease in counts, respectively, in regions where the milk was more acidic and nutrient levels were lower (Yabrir *et al*., 2013). Some insight into the microbiology of sheep milk was also provided by a recent study of the raw sheep milk cheese, Oscypek, which is manufactured without a starter culture (Alegria *et al*., 2012; Table 3). As this is a naturally fermented raw milk cheese, it is likely that these cheese-associated bacteria were also present in the corresponding raw milk. A culture-based approach established that *lactococci* (*Lactococcus lactis ssp. lactis* and *ssp*. cremoris) dominated (c. 109 CFU g 1),

with lactobacilli (*Lactobacillus casei, Lactobacillus plantarum, Lactobacillus parabuchneri* and *Lactobacillus brevis*) also being common (107–108 CFU g 1). *Leuconostoc* (*Leuconostoc citreum, Leuconostoc lactis and Leuconostoc mesenteroides*) were detected at levels of 10^5 10^8 CFU g 1, fungal populations were present between 10^5 and 10^6 CFU g 1 and Enterobacteriaceae, including *Enterobacter kobei*, were at 10^3–10^6 CFU g 1, but were reduced during processing. A parallel DGGE investigation confirmed the dominance of *Lactococcus lactis* but also highlighted the presence of a significant population of *Lactococcus garvieae*, which had not been detected by culturing. This approach also revealed a number of minor populations including *Tetragenococcus halophilus, Streptococcus salivarius, S. thermophilus* and *Streptococcus vestibularis.* A high-throughput sequencing–based approach revealed the presence of 40 different genera in the cheese. This included 9 dominant genera, including 6 from the order *Lactobacillales* (which include the *lactococci,* lacto bacilli and related genera), which constituted 97% of assigned sequences. The other dominant genera were the *Bifidobacteriaceae, Enhydrobacter* and unclassified Bacilli. The benefits of employing this technology were again highlighted when previously overlooked populations.

Buffalo milk

Buffalo milk is consumed in various countries around the world, with India and Pakistan being the highest consumers. It is not as common in Europe, but it does have an important market in some Mediterranean countries where it is utilized in making traditional mozzarella cheese. The microbial content of raw buffalo milk has been assessed, through culturing, and found to contain a large population of LAB, including *lactococci and lactobacilli,* as well as coliforms, *E. coli, S. aureus* and bacterial endosp ores, highlighting that while technologically relevant bacteria are present, microorganisms of concern with respect to quality and safety can also be found (Ercolini *et al*., 2004; Han *et al*., 2007). Culture-independent methods, that is, DGGE, have revealed that raw buffalo milk contains a rich diversity of bacteria that changes during subsequent fermentation to manufacture traditional mozzarella (Ercolini *et al*., 2001). More recently, high-throughput sequencing has been applied to identify the bacterial populations present in buffalo milk and throughout the manufacture of mozzarella cheese (Table 3; Ercolini *et al*., 2012). The dominant microorganisms in the milk were *Lactococcus spp.* (30%), *Acinetobacter spp.* (21%), *Pseudomonas spp.* (20%), *Streptococcus macedonicus* (10%) and *Lactococcus lactis* 10%. A number of other microorganisms were detected in low abundance including

Brochothrix, Carnobacterium, Chryseobacterium, Clostridium, Corynebac terium, Enterobacteriaceae, Gammaproteobacteria and *Haloanella*. There was also a large percentage of unassigned reads (c. 20%) corresponding to the raw milk. This percentage was much greater than that associated with the corresponding cheese (Ercolini *et al*., 2012).

Materials and Methods

Lactating dairy cows, Goat, buffalo were selected for the present study.

Procedures for Collecting and Handling Samples. For the bacteriological analysis, raw milk samples from dairy farms, and households. A total of 300–500 ml of milk and milk products were collected from dairy farms, individual households from farmers by using a sterile glass bottle. The samples were labeled correctly, stored at 4^{o} C, and the samples were cultivated bacteriologically.

Aerobic Plate Count The aerobic plate count (APC) standardized method of International Organization for Standardization (ISO- 4833-1:2013) [11] was used. One millilitre of the raw milk sample was aseptically added to a test tube with 9ml sterile 0.1 % peptone water and serially diluted, using the ten-fold dilution technique. One millilitre of the final diluents was aseptically pour-plated into properly labeled sterile petri- dishes, with moderately cooled liquified plate count agar and the plates swirled to mix properly. This experimental process was carried out in duplicates. The plates were incubated in inverted position for 24 hours at 37 °C. Afterwards, the plates were observed for colony growths and the obtained colonies counted and recorded as colony forming units (CFU/mL). Plates which had 25 – 250 colonies were counted. The mixed colony cultures were separately and repeatedly sub-cultured on fresh agar plates until pure colonies were obtained. The pure isolates were grown on agar slants and refrigerated until when needed [12].

Enterobacteriaceae, Salmonella, Shigella, Staphylococcus and *Pseudomonas* counts, The official technique of the Association of Official Analytical Chemists (AOAC) International, as described by Latimer [13], was used to to isolate enteric bacteria from raw cow milk. One millilitre of the milk sample was added into a test tube containing 9 ml sterile 0.1 % peptone water and the test tube incubated overnight for pre-enrichment. Afterwards, the incubated broth was serially diluted with the aid of the ten-fold technique. The final diluent was pour-plated, separately with cooled molten agars of eosin methylene blue (EMB), xylose lysine deoxycholate (XLD), cetrimide and mannitol salt into carefully labelled petri dishes for *Enterobacteriaceae, Salmonella, Shigella, Pseudomonas and*

Staphylococcus counts, respectively. This experimental process was carried out in duplicates and the plates incubated at 44.5 °C. for 24 hours. Plates with 25 – 250 colonies were counted.

After incubation, colonies were counted and recorded as colony forming units (CFU/mL). Morphologically distinct colonies were sub-cultured and purified by streaking on appropriate agar plates repeatedly until pure colonies were obtained. 2.4 Aerobic spore-forming bacteria count The isolation and bacterial count techniques described by Ryu et al. [14] was utilized. A total of 5 ml of the raw milk was heated in a water bath at 80 °C for 10 minutes. Then, 1 ml of the milk was serially diluted in test tubes with 9 ml of 0.1 % sterile peptone water and the diluents pour-plated with molten nutrient agar into sterile petri dishes. The plates were incubated at 37 °C for 24 hours. The plate counts were expressed as CFU/ml.Isolation and Identification of microbial flora.

Identification of obtained isolates Each obtained isolate was subjected to conventional biochemical identification keys and tests such as Gram staining, catalase, coagulase, methyl-red, Voges-Proskauer, Indole, starch hydrolysis, sugar fermentation and endospore staining. The isolates were identified as described in Bergey's Manual of Systematic Bacteriology [15]. 2.6 Statistical analysis The differences in means among the various bacterial counts obtained were determined with the one-way analysis of variance (ANOVA), followed by Duncan's multiple range test, using the Statistical Package for Social Sciences (SSPS software, IBM version 20).

Results

Bacterial count The different bacterial counts of raw cow milk obtained from the bulk tank is shown in Table 1. During the sampling process, the aerobic plate counts ranged from 6.0 x 105 (5.78 log CFU/ml) to 8.0 x 10 9 (9.90 log CFU/ml), while the mean aerobic plate and geometric mean counts obtained were 8.21 log CFU/ml and 1.61 x 108 CFU/ml, respectively. The *Enterobacteriaceae, Salmonella-Shigella, Staphylococcus, pseudomonas spp,* and aerobic sporeformers counts obtained in this study are also shown in Table 1. The mean log counts of 5.8±1.12 CFU/ml, 8.8±0.69 CFU/ml, 8.1±0.48 CFU/ml, 7.1±0.48 CFU/ml and 6.1±0.73 CFU/ml, respectively, were obtained.

Table 1: Bacterial counts (CFU/ml) obtained from raw milk in bulk-tank storage

Type of Count	Sample	CFU/ml	Log CFU/ml	Mean Log CFU/ml	Geometric Mean (CFU/ml)
Aerobic plate	1	6.0×10^5	5.8	8.2±1.71	1.61×10^8
	2	8.0×10^9	9.9		
	3	7.0×10^7	7.9		
	4	2.0×10^9	9.3		
Enterobacte-riaceae	1	4.2×10^6	6.6	5.8±1.12	-
	2	8.0×10^5	5.9		
	3	1.0×10^4	4.0		
	4	3.2×10^6	6.5		
Salmonella/ Shigella	1	9.0×10^7	7.9	8.8±0.69	-
	2	4.8×10^8	8.7		
	3	4.2×10^8	8.6		
	4	6.0×10^9	9.8		
Staphylococcus	1	7.0×10^7	7.8	8.1±0.48	-
	2	2.8×10^8	8.4		
	3	5.1×10^8	8.7		
	4	4.0×10^7	7.6		
Pseudomonas	1	4.1×10^7	7.6	7.1±0.48	-
	2	2.5×10^6	6.3		
	3	2.0×10^7	7.3		
	4	1.2×10^7	7.0		
Aerobic spore-formers	1	2.0×10^5	5.3	6.1±0.73	-
	2	4.0×10^4	5.6		
	3	1.2×10^7	7.0		
	4	2.0×10^6	6.3		

Table 2 reflects the assessment of the different mean log bacterial counts (CFU/ml) from raw milk. Results revealed three subsets of significant differences were observed in the various mean counts obtained. The mean *Enterobacteriaceae* and aerobic spore counts were homogenous, and likewise the mean Staphylococcus spp., aerobic plate and *Salmonella-Shigella* counts. Nevertheless, these two groups of means significantly differed from each other, while the mean *Pseudomonas* count, was also significantly different from the aforementioned groups.

Table 2: Duncan's multiple range test assessment of the different mean log bacterial counts (CFU/ml)

Bacterial count	Mean log count (CFU/ml)
Enterobacteriaceae	5.8 ±1.2[a]
Aerobic spore formers	6.1 ±0.73[a]
Pseudomonas	7.1 ±0.48[b]
Staphylococcus	8.1 ±0.48[c]
Aerobic plate	8.21± 1.71[c]
Salmonella—Shigella	8.8 ±0.69[c]

Means with same superscripts in a column are not significantly different (P<0.05)

Isolated bacteria A summary and occurrence of the different bacteria genera isolated from raw milk in bulk tank storage is shown in Table 3. Out of the 73 bacterial isolates obtained from the study, the microflora in raw cow milk included *Enterobacter spp* (24), *Staphylococcus aureus* (2), *Staphylococcus epidermidis* (10) *Pseudomonas spp* (6), *Salmonella spp* (9), *Shigella spp* (5), *Bacillus spp* (9), *Paenibacillus spp* (5) and *Enterococcus spp* (3),

Type of Count	Sample	CFU/ml	Log CFU/ml	Mean Log CFU/ml	Geometric Mean (CFU/ml)
Aerobic plate	1	6.0×10^5	5.8	8.2±1.71	1.61×10^8
	2	8.0×10^9	9.9		
	3	7.0×10^7	7.9		
	4	2.0×10^9	9.3		
Enterobacte-riaceae	1	4.2×10^6	6.6	5.8±1.12	-
	2	8.0×10^5	5.9		
	3	1.0×10^4	4.0		
	4	3.2×10^6	6.5		
Salmonella/ Shigella	1	9.0×10^7	7.9	8.8±0.69	-
	2	4.8×10^8	8.7		
	3	4.2×10^8	8.6		
	4	6.0×10^9	9.8		

Discussion

In this study, the total aerobic plate count was 8.21 log CFU/ml. This similarly compares with the total APC of 8.149 log CFU/ml obtained from raw cow milk in Borena Yabello, South Ethiopia [16]. However, lower APC values of 6.76 log CFU/ml [17] and 6.01 log CFU/ml [18], respectively, have also been reported. It is worth noting that the limit of

acceptable total APC in raw milk is 5.0 log CFU/ml [19]. APCs higher than the acceptable limit could reflect poor sanitary quality of raw milk as well as poor conformance with good manufacturing practices during production; these would in turn affect the sensory acceptability of the milk, and the shelf-life of the derivative dairy products. Nonetheless, APC is not an index of food safety as there is no direct correlation to occurrence of pathogen or toxin, just as low APC does not signal absence of pathogens in a product [20]. Geometric mean expresses the impact of the upper allowable microbiological count limits for raw milk rather than the arithmetic mean [21], since it decreases and smoothens variation in high and low sampling count often encountered while taking

Enterobacteriaceae are the significant causes of serious infections, since these Gram-negative bacteria can directly or in directly penetrate into food and can persist in the environment as secondary contaminants. Occurrence of a high Enterobacteriaceae count in raw milk is not frequently considered as a reliable indication of fecal contamination or enteric pathogens as there are several *Enterobacter* species of non- fecal origin [22].

The mean Enterobacteriaceae count of 5.8$\pm$ 1.12 log CFU/ml indicated in this study showed a relatively high count but still falls within the range 0 -10.18 log CFU/ml reported by Ibtisam *et al* [23] for *Enterobacteriaceae* counts in raw milk from Khartoum State, Sudan, and higher than the mean count range of 4.67 – 4.95 log CFU/ ml asserted by Pyz-Lukasik et al [24] of raw milk in Poland. Enterobacteriaceae are present in raw milk because of secondary bacterial contamination during milking, and this count is a helpful predictor of this production-stage cleanliness [24]. The presence of Enterobacteriaceae, if allowed to persist in dairy products, induces undesirable changes that could render the product of inferior quality, unmarketable, and unfit for human consumption [20].

In this study, the mean *Staphylococcus* spp. count derived from the milk samples collected was 8.15 log CFU/ml. which is relatively higher than the count range of between 3.2- 4.7 log CFU/ml reported by Dai *et al*. [25] in China. Higher S*taphylococcus* spp count of up to 9.32 log CFU/ml has also been reported in cow milk from Egypt [26].

Staphylococcus aureus and *Staphylococcus epidermidis* were the predominant *Staphylococcus* species identified in this study. This is not surprising because factors such as, improper sanitation of milking equipment, sub-optimal hygiene of milking personnel, incorrect udder preparation, poor farm hygiene, milk transportation without cold chains, and a lack of knowledge of food-borne illnesses have been associated with Staphylococcal

contamination [27]. Mastitis is another additional reason for the high pooled prevalence of *Staphylococcus* spp in raw cow milk [28].

Staphylococcus spp can be expelled directly from the mastitis udder into milk and afterward contaminate bulk milk and raw milk products [29]. Mixing of milk from different farms including mastitis milk, can also contribute to higher contamination of raw cow milk with *Staphylococcus* spp [30].

The mean aerobic spore forming bacteria count obtained in this study was 6.1±0.73 log CFU/ml. Milk and dairy products are susceptible to a variety of spoilage microorganisms, but the spore-forming bacteria are particularly dangerous. Their spore-forming ability allows them to endure the harsh dairy processing conditions, hence, survive and proliferate in the dairy environments [31]. Spore-forming bacteria are widely distributed in the soil; which serves as the main contaminant source [32]. Other possible sources of contamination are feaces, bedding, feed or milking equipment [33]. Furthermore, they can get into raw milk and onwards to dairy processing facilities via contaminated teats, milking cups, bulk tanks or via transportation [34].

Aerobic spore-forming bacteria such as *Bacillus* spp and related bacterial species are the most dominant and widely proliferated groups in the dairy industry. This study has identified *Bacillus* spp and *Enterococcus* species as part of the bacteria encountered. These classes of genera have a strong correlation

with pathogenicity and activities that cause spoiling in milk and dairy products [35]. Another spore- forming genera obtained in this study was *Paenibacillus* spp. These species of spore formers can survive at both high temperatures as well as the refrigeration temperatures. *Enterococcus* species are highly valued, important mixed starter culture components in cheese production, and particularly useful for the development of taste and flavour during cheese ripening [36]. On the other hand, these same species have been considered undesirable in the food industry because there is an opinion that these organisms predominate in the gastrointestinal tract of humans and animals; their presence are indicators of fecal pollution and could transmit antibiotic-resistance genes and virulence factors [37]. Nevertheless, the presence of *enterococci* in raw milk is not necessarily connected to fecal contamination, as these organisms can enter food from other sources, such as water, animal feed, fomites used in the milking process or the animal's exterior [38]. *Enterococci* are ubiquitous in the environment and can be present in raw milk without being of fecal origin [39]. The Federal

Food, Drug, and Cosmetic Act of the United States has not proposed GRAS status for members of the genus *Enterococcus* because of their controversial epidemiological status [40].

In this study, the mean bacterial counts for *Pseudomonas* spp and *Salmonella-Shigella* spp were 7.1 ±0.48 log CFU/ml and 8.8 ±0.69 log CFU/ml, respectively. Many strains of *Pseudomonas spp* possess psychrotrophic characteristics [41]. This attribute may explain the high counts obtained for these organisms as they could survive under the low storage tank temperature. In addition, they produce a number of exoenzymes that can contribute to the deterioration of raw milk, hence, are food spoilage organisms [42]. Similarly, Salmonella spp. can attach to various materials, during their life-cycle, produce biofilms and contaminate the food chain; hence, become a potent threat to the health of consumers [43].The presence of *Pseudomonas* spp and *Salmonella*/*Shigella* spp indicate that safety precautions be adopted while processing the milk. Water, feaces (human and animal), equipment and personal hygiene practices are known as common sources for these group of bacteria [43].

Bacteria such as *Enterobacter*, *Enterococcus*, *Staphylococcus*, *Pseudomonas* spp. have been commonly related with cow milk [44] and these include bacteria found in the gut and skin of a cow, pathogens, psychrotrophs, helpful and spoilage bacteria [45]. These spectra of organisms were isolated from raw milk in this study.

Conclusion

This study has shown that 8.12±1.71 log CFU/ml, 5.8±1.2 log CFU/ml, 7.1±0.48 log CFU/ml, 8.1±0.48 log CFU/ml, 8.8± 0.69 and 6.1±0.73 log CFU/ml were the mean APC, *Enterobacteriaceae*, *Pseudomonas* species, *Staphylococcus* species, Salmonella-Shigella and aerobic spore-forming bacteria counts, respectively, obtained from raw bovine milk stored in a bulk-tank. The outcomes reveal high bacteriological contamination which apparently reflect differences in dairy farm practices, high environmental temperature, transportation and personal hygiene habits. Nevertheless, it is suggested that periodical training and retraining local dairy farmers towards developing a consciousness and esteemed value to the fact that microbial load of milk is an important component in determining milk quality and in producing safe milk for consumers. Hence, concerted efforts should be stirred towards minimizing bacterial recontamination of raw milk prior to storage in a collecting centre.

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