

Chapter One

INTRODUCTION

1.1 General background

Man has been using animal milk for his food 4000 years B.C. Qur'an statements and Suna mentioned the benefits of milk. Sudan is a large country with different climatic conditions which enable it to be the largest country in the number of animal. According to the international classification it is the 1st in the number of camel (3.54 million head), the fourth in the number of goats (43.14 million head) ,the sixth in the number of cattle(40 million head)and the seventh in the number of (sheep 49.05) (Sudanese Standard and Metrology Organization - SSMO, 2007).

The dromedary camel (*Camelus dromedaries*, one-humped camel) is the most important livestock animal in the semi-arid areas of Northern and Eastern Africa as well as in the deserts of the Arabian Peninsula. It is a multipurpose animal, used for its supply of milk, meat, hides and transport (Burgemeister, 1974; Kappeler, 1998).

Camel milk is one of the most valuable food resources for nomads in arid regions and can contribute to a better income for pastoralists, especially as in the last years milk consumption among the urban population was increasing (Farah, 2004; Chaibou, 2005).

The one humped camel is an essential source of meat and milk in many parts of the world and especially in developing countries in Africa and Asia. The dromedary plays also economic, social and ecological roles (Warden, 1992; Ouajd and Kamel, 2009).

The fact that camel milk is mainly consumed in its raw state (boiling of the milk is not common as it is known to remove its “goodness”), the high ambient temperature and the lack of refrigeration facilities in many arid areas are the main reasons for hygienic problems (Radwan *et al.*, 1992; Semereab and Molla, 2001).

Milk is an ideal food for human being irrespective of ages and undoubtedly the most important one among the foods of animal origin (Boscos *et al.*, 1996).

Milk is an ideal habitat for the growth and multiplication of microorganisms due to its nutritional constitution which contain protein, carbohydrate, minerals and vitamins. All these components support the growth of many forms of bacteria (Omer and Eltinay, 2008).

Milk is a complex biological fluid and by its nature, a good growth medium for many microorganisms. Because of the specific production it is impossible to avoid contamination of milk with micro-organisms, therefore the microbial content of milk is a major feature in determining its quality (Rogelj, 2003).

Raw camel milk may contain microorganisms pathogenic for man and their source may lie either within or outside the udder (Sinell, 1973). Many epidemiologists reports proved that, non-heat treated milk and raw-milk products represent the major factors responsible for illnesses caused by food borne pathogens (De Buyser *et al.*, 2001).

El-Ziney and Al-Turki (2007) reported that, approximately 50% of the examined raw camel's milk samples were produced and handled under poor hygienic conditions with high health risk to the consumers. Bacterial and somatic cell counts are reference methods used as indicators of raw milk quality (Costello *et al.*, 2003).

Microbial contamination of milk can generally occur from three main sources (Mckinnon *et al.*, 1990); from within the udder, the exterior of the udder and the surface of milk handling and storage equipment. All these sources of contamination influence the Total Bacteria Count (TBC) or Standard Plate Count (SPC).

When regulatory standards for bacterial counts in raw milk are met, pasteurization is highly effective in destroying pathogenic microorganisms that can present a threat to human health (Boor and Murphy, 2002).

Bacterial contamination of raw milk can originate from different sources: air, milking equipment, feed, soil, faeces and grass (Coorevits *et al.*, 2008). The number and types of micro-organisms in milk (immediately after milking) are affected by factors such as animal and equipment cleanliness, season, feed and animal health (Rogelj, 2003).

It is hypothesized that differences in feeding and housing strategies of milking animals may influence the microbial quality of milk (Coorevits *et al.*, 2008). Rinsing water for milking machine and milking equipment washing also involve some of the reasons for the presence of a higher number of micro-organisms including pathogens in raw milk (Bramley, 1990). After milking, milk is cooled, which additionally influence the dynamic of microbial process (Rogelj, 2003).

Most of the consumers in Sudan use raw camel milk without cooling as the marketing system of milk is a transitional one (Elmagli and El Zubeir, 2006).

1.2 Objectives:

1.2.1 General objectives:

To investigate the occurrence and presence of bacteria in raw camel milk with special reference to food borne pathogens.

1.2.2 Specific objective:

- (i)** To isolate and identify different bacterial species from raw camel milk in Khartoum State.
- (ii)** To estimate the total viable bacterial count of raw camel milk sample from different localities.
- (iii)** To assess the possible hazards that may occur as a result of consuming unpasteurized camel's milk.

Chapter Two

LITERATURE REVIEW

2.1 Importance of camels:

The camel is an important livestock specie uniquely adapted to the hot environment (Schwartz and Dioli, 1992a). Moreover, it is an ideal domestic animal in the desert with long, dry, hot periods of eight months or more and scarce annual rain falls between 50 and 500 mm (Ramet, 2001). Camels are the only reliable milk producer due to their unique adaptation to hot and arid environment (Elamin, 1979 and Schwartz, 1992b). One of the most remarkable features of dehydrated camels is the ability to continue lactation and to secrete milk that is highly diluted with over 90% water content (Mohammed, 2006). In true ruminants the reservoir for milk water is lost for cooling and via fecal and urinary excretion. In cattle, sheep and goats, the lack of water leads to cessation of lactation or to a very concentrated high fat and low water content milk. A 600 kg camel has about 200 liters of fluid in the alimentary tract, which is available for milk production, giving 20 liter per day for 10 days. Lactating camels, therefore, will guarantee ample food with the desired content for their off-spring and humans alike (Yagil, 2000).

Camels are better suited than goats and sheep for animal husbandry in desert areas (Elamin, 1979). Unlike sheep and especially goats, which chew every piece of vegetation to the roots and denude areas around oases, camels take only a few bites from a shrub and a bush and then move on. Hence they are true browsers, thus not destroying their habitat (Wernery, 2003).

The watering intervals for the camel in Sudan vary from 7-9 days during summer and 20 - 30 days during winter (Musa *et al.*, 2006).

It has been reported that camels can be managed in closed farms where they produce a high quality food product; the milk. Unfortunately, very few countries produce camel milk commercially (Wernery, 2003).

Camel milk has the ability to inhibit the growth of pathogenic microorganisms because it contains enzymes with anti-bacterial and anti-viral properties (Elagamy *et al.*, 1992).

Lactoferrin: Prevents microbial growth in the gut, the amount is higher in camel milk than in cow milk.

Lactoperoxidase: Contributes to the non immune host defense system, suppresses Gram-negative bacteria, it is most effective in raw milk during the first 4 days.

Peptidoglycan recognition protein (PGRP): broad antimicrobial activity, stimulates the immune system effect on breast cancer, is higher in camel milk than in cow milk.

N-acetyl-glucosaminidase (NAGase): anti-bacterial activity and antiviral activity.

2.2 Taxonomy and breeds:

In zoological taxonomy, camelids are classified in the suborder Tylopoda (pad-footed animals) that represents with the suborders Suiformes (pig-like) and Ruminantia (ruminants) the order Artiodactyla (even-toed ungulates). This makes obvious that camelids (family Camelidae) as ruminating animals are classified in proximity to ruminants but developed in parallel and are not part of the suborder Ruminantia. Some differences as foot anatomy, stomach system and the absence of horns underline this fact (Schwartz and Dioli, 1992a; Fowler, 1998; Wernery, 2003).

The family Camelidae is divided into three genera: The old world camels (genus *Camelus*) and the new world camels (genus *Lama* with the species *L. glama*, *L. guanicoe*, *L. pacos* and genus *Vicugna* with the species *V. vicugna*) (Wilson and Reeder, 2005). Legel (1990) described only two genera (*Camelus* and *Lama*) Two domesticated species of old world camels exist: the dromedary or one humped camel (*Camelus dromedarius*, Table 1) that has its distribution in the hot deserts of Africa and Asia and the Bactrian or two-humped camel (*Camelus bactrianus*) that can be found in the cold deserts and dry steppes of Asia. In the desert Gobi there is still a population of wild two-humped camels classified as *Camelus ferus* (RAO *et al.*, 1970; Peters, 1997; Fowler, 1998).

The Bactrian camel was named after the area of Bactriana in Central Asia. The name of the dromedary has derived from the Greek word “dromeus” which means runner or “droma” - running (Jassim and Naji, 2002). The one-humped camel was probably domesticated in the region of today’s Yemen and Oman about 3000 to 4000 years ago (Fowler, 1998). The wild Arabian camel became extinct (Lensch, 1999).

Table 1 : Genealogy of the dromedary camel (Wilson, 1984)

Order	Artiodactyla (even-toed ungulates)
Suborder	Tylopoda (pad-footed animals)
Family	Camelidae
Subfamily	Camelinae
Genus	Camelus
Species	<i>Camelus dromedaries</i>

2.3 Camel population in the world:

According to FAO statistics (Global Livestock Production and Health Atlas - GLIPHA, 2006) the world population of camels is about 20 million animals, mainly in arid zones, of which 15 million camels live in Africa and 5 million in Asia (GLIPHA, 2006). In 2001, the total camel population was 19 million of which 17 million were dromedaries (*C. dromedarius*) and 2 million were Bactrian camels (*C. bactrianus*) (Farah, 2004).

2.4 Characteristics of lactation and camel milk :

2.4.1 Anatomy of the camel udder

The camel udder consists of four quarters, each with two, sometimes three separated glandular complexes leading into one teat. So in each teat there are two (or three) milk canals. (Yagil, 1985).

The left and right halves of the camel udder are separated by a groove as the udder is suspended by fibro-elastic tissue, leading from the linea alba to the prepubic tendon (Smuts and Bezuidenhout, 1987). As one-humped camels are not systematically bred for milk production, there is a great variety in different udder

and teat shapes and sizes. Additionally the shape can vary according to age and stage of lactation (Albrecht, 2003; Wernery and Kaaden, 2004).

2.4.2 Characteristics of camel milk:

Camel milk has a white opaque colour, a faintly sweetish odour and a salty taste (RAO *et al.*, 1970) due to the type of plants eaten in the desert by the camels (Khaskheli *et al.*, 2005). The changes in taste are mainly caused by the type of fodder and availability of drinking water (Farah, 1996). It is thinner than cow or buffalo milk (Ohri and Joshi, 1961; Abdurahman, 1996a). A camel milk has a much slower natural creaming rate than cow milk - in its raw state and heat treated (Farah and M. Rüegg, 1991; Farah, 1993). Camel milk is frothy when slightly shaken (Shalash, 1979).

2.4.3 Nutritional quality of camel milk

The ability of the camel to survive in semi arid condition make it an important source of food in drought areas of the world where famine is endemic to over 20 million people each year (Yagil *et al.*, 1984). The camel herders relay completely on camels milk for more than a month without having drinking water (Bakheit, 1999).

The most important factor in camel milk for peoples living in dry zones is its water content (Wilson, 1998). Yagil (1982) declared that young camel and human living in dry areas are in need of fluids to maintain homeostasis and thermoregulation. The pastoralists usually relay on camel milk throughout the year (Bakheit, 1999 and Igbal, 2002) and it may contribute up to 50% of their food (Chabeda, 2002).

Camel milk is known to be a rich source of vitamin C, the vitamin content was three times (Farah *et al.*, 1992) to five times (Stahl *et al.*, 2006) higher than that in bovine milk. Hence, raw and fermented camel milk could be a good source of vitamin C for the people living in the desert area where vegetables and fruits are not available (Sawaya *et al.*, 1984). The mean of vitamin C content in Dromedary camel milk is 34.16 mg/ l (Sawaya *et al.*, 1984; Farah *et al.*, 1992 and Haddadin *et al.*, 2008). Compared with bovine milk, the niacin (B3) content was reported to be higher in camel milk (Sawaya *et al.*, 1984 and Haddadin *et al.*, 2008). The content of vitamin A and riboflavin (B2) in Dromedary camel milk was reported to be lower than that of bovine milk (Sawaya *et al.*, 1984; Farah *et al.*, 1992 and Stahl *et al.*, 2006). Whereas the concentration of vitamin E was very close to that of bovine milk (Farah *et al.*, 1992). The amount of the major minerals is similar in camel and cow milk. nevertheless, variations exist due to different feeding practices. As they found low concentrations of copper and iron in dromedaries of the Arabian peninsula (Wernery *et al.*, 2002).

2.4.4 Medicinal benefits of camel milk

Yagil (1982) mentioned that camel milk is given to the sick, elderly and the very young because of the belief that it is not only healthier, but also works especially well in bone formation.

Research indicated that camel's milk has many properties that make it very useful to consumers as camel's milk used in some parts of the world to cure certain diseases (Knoess, 1982; Yagil, 1982 and Wernery, 2003). Attia *et al.* (2001) and Sarwar (2002) reported that camel milk has medicinal value. Yagil (1982) stated that nomads belief on that any internal disease can be cured by drinking camel

milk and for this reason camel milk is being used therapeutically against dropsy, jaundice, problem of spleen, tuberculosis, asthma and biles (RAO *et al.*, 1970) and cancer (Wernery, 2003). RAO *et al.* (1970) reported that patient with chronic hepatitis had improved liver function after being treated with camel milk. However they mentioned that camel milk also works as laxative for people unaccustomed to drink this milk.

Wernery (2003) reported that camel milk includes insulin and therefore can be used to treat diabetes. Moreover (Agrwal *et al.*, 2005) cited that camel milk appear to be safe and effective in improving long term glycemia as it controls and helps in reduction of the insulin for patient with type 1 diabetes.

Camel's milk is stated to have health properties, that all bacteria are driven from the body. Moreover this is to be true only for camels that eat certain shrubs and bushes, which, themselves, are used in the preparation of medicine, however, camel that eat straw are said to lose this ability (Yagil, 1982).

2.5 Bacteria in camel milk:

Milk is a good medium for several bacteria to develop. The growth of bacteria in milk depends mainly on temperature and the presence of other bacteria (Heeschen, 1994). As camel milk is usually consumed in its raw state, the presence of pathogenic bacteria may be of public health importance besides its influence on animal health (Saad and Thabet, 1993; Younan, 2004). Generally, bacteria in milk can occur through colonisation of the teat canal or an infected udder (clinical or subclinical mastitis) or as contaminants.

Milk is synthesized in specialized cells of the mammary gland and is virtually sterile when secreted into the alveoli of the udder (Tolle, 1980 and Marth, 1985). (Murphy and Boor, 2000) reported that as milk leaves the udder of healthy animal, normally contains very low number of microorganisms.

Measurement of bacterial number in milk is of interest, because they are indicators of poor milk hygiene (Harding, 1999). Because of its properties, camel milk bacteriology is relevantly different in comparison to milk from other species (Semereab and Molla 2001 and Karimuribo *et al.*, 2005). The total bacterial count of camel milk is reported with values that vary between 10^2 and 10^8 cfu/ml. In Ethiopia the range was reported as 4×10^5 (Semereab and Molla, 2001), in UAE, $94.1 \% < 1.0 \times 10^5$ (Wernery *et al.*, 2002), in Kenya $10^3 - 10^5$ (Younan., 2004) and the mean total bacterial count of samples collected from two locations of Khartoum State was 1.22×10^8 (Shuiep *et al.*, 2007).

2.5.1 Milk Bacterial Diseases:

Zoonotic Infectious diseases of food safety importance:

The zoonotic risk arising from camel milk should be considered because camel milk is usually consumed in its raw state (Radwan *et al.*, 1992; Younan, 2004).

2.5.1.1 Brucellosis:

Brucellosis is one of the most important zoonoses and affects human welfare and livestock health worldwide. It exists especially in the Mediterranean Basin, the Arabian Peninsula, the Indian Subcontinent and parts of Central and South America. The disease is caused by bacteria of the genus *Brucella* which includes

different species (mainly *B. abortus* and *B. melitensis*) that vary in their affinity and virulence to several hosts (FAO, 2004a; FAO, 2004b).

Old world camels are susceptible to *B. abortus* (bovine brucellosis) and *B. melitensis* (ovine/caprine brucellosis) (Strauss, 1995; FAO, 2004a). Both may cause widespread animal health problems in the Arabian Peninsula, occurring regularly in the UAE, as well as in Saudi Arabia, Yemen, Qatar, Kuwait and since 2002 also in Bahrain (OIE, 2004; OIE, 2006). Yet no human cases were reported in the last 9 years (OIE, 2004). However, several reports exist describing a human infection caused by consuming fresh camel milk. (Burgemeister *et al.*, 1975) found the presence of *B. abortus* antibodies of 7.7 % in dromedaries in Tunisia, whereas Teshome and Molla (2002) proved a total seroprevalence of *B. melitensis* in camels of 5.9 % in different regions of Ethiopia. Also Radwan *et al.* (1992) and Wernery *et al.* (2007a) reported a seroprevalence of *B. melitensis* in camels in Saudi Arabia and the UAE. As camel milk is often consumed in its raw state, the presence of *Brucella spp.* has to be taken as a serious health risk even if it seems that the excretion rate of *Brucella* organisms is lower than in goats and these organisms are not capable of growing in milk (Heeschen, 1994; Younan, 2004). Epidemiologically, brucellosis in camels seems to be related to the prevalence of *B. melitensis*. According to Younan (2004) it appears that there is a clear correlation between infections of sheep and goats with *B. melitensis* and infections of camels. In the above described study farmers and milkers were examined with the result that 20 % of them showed Malta fever due to *B. melitensis*.

2.5.1.2 Bovine tuberculosis:

Is a chronic disease caused by bacteria of the genus *Mycobacterium* that affects many animal species. It is characterized by development of tubercles in the organs of most species. Bovine tuberculosis is caused by *M. bovis* and is a significant zoonotic disease (FAO, 2004d). As *M. bovis* is inactivated by pasteurisation,

mainly raw camel milk plays a role in transmission of tuberculosis to humans (FAO, 2004d; Younan, 2004), even if *M.bovis* is not capable of growing in milk. This can be the case, if camels are kept in close contact to other livestock sensible to tuberculosis (EFSA, 2003; FAO, 2004d). In camel necropsy examinations *M. bovis*, *M. avium* and *M. kansasii* were isolated (Strauss, 1995). One outbreak of tuberculosis in camels caused by *M. bovis* has been reported since 1996 in the UAE (Wernery *et al.*, 2007b). Bovine tuberculosis is also endemic in Bahrain (last confirmed case in 2003) and Qatar (last confirmed case in 2002) (OIE, 2004; OIE, 2006). In 2006 one case of camel tuberculosis caused probably by a representative of the *M. africanum* subtype 1 has been described by (Kinne *et al.*, 2006).

2.5.1.3 Para tuberculosis (Johne's disease):

M. avium subsp. paratuberculosis is of worldwide concern in milk production due to the issue of its potential role in Crohn's disease in humans. An investigation of raw bulk milk samples and pasteurised cow milk in the United Kingdom showed, that *M. paratuberculosis* is occasionally present in raw and correctly pasteurised cow milk (72 - 74 °C for 25 s, phosphatase-negative) (Grant *et al.*, 2002). Few is known about paratuberculosis in camels but infections with *M. avium* subsp. paratuberculosis are reported in old world camels (Burgemeister *et al.*, 1975; Fazil and Hofman, 1981; Kinne *et al.*, 2007). According to OIE (2004) and OIE (2006) the last confirmed case of paratuberculosis in the UAE and in Oman occurred in 1999 in ovines, however, one male dromedary in the UAE died from camel paratuberculosis and represents the first case in camels in this country for 13 years (Kinne *et al.*, 2007).

2.5.1.4 Q fever:

Q fever is an infectious disease caused by *Coxiella burnetii*. It is of public health importance as it can be transmitted to humans by milk - frequently milked from clinically inapparent domesticated animals, but it is inactivated by pasteurisation (FAO, 2004c). *C.burnetii* seems to be wide-spread in camels according to (Strauss, 1995). This complies with the findings of (Burgemeister *et al.*, 1975) who proved 17.3 % of serological positive in Tunisia. Some non-confirmed cases of Q fever in animals have been reported in Bahrain from 1997 - 2000 and in Oman 2003 and 2004. No case in the UAE has been reported in the last years (OIE, 2004; OIE, 2006).

2.5.2 The main source of contamination of raw milk

Generally the microbial contamination in raw milk occurs from within the udder, exterior of the udder and the surface of the milk handling equipment (Murphy and Boor, 2000). Moreover the skin of the udder, tick wounds on the teats and milker's hand, especially if unwashed perfectly before milking or with wounds, are among the sources of contamination (Kenyanjui *et al.* , 2003). Besides, the dust and flies at the milking site, especially if milk containers were left open. Also the use of unclean water for milking process are among the sources of contamination (Omar, 2003). Under pastoral production conditions, environmental contamination is likely to play a bigger role in the hygiene of raw camel milk than initial bacterial contamination of the camel milk (Younan, 2004).

Pathogenic bacteria may also be present in raw camel milk as direct consequence of udder disease (Murphy and Boor, 2000), especially mastitis (El

Zubeir and ibtisam, 2003). Mastitis pathogens, as far as they are zoonotic, are of public health concern as some of them are capable of producing toxins or causing infections in man (Semereab and Molla, 2001).

Table 2 : Average TBC in camel milk

Author(s)	Country	TBC (cfu/ml)
Semereab and Molla, 2001	Ethiopia	$4 \times 10^5 - 10^5$
Wernery <i>et al.</i> , 2002	UAE, bowl samples	94.1 % < 1.0×10^5
Younan, 2004	Kenya, udder samples	$10^2 - 10^4$
Younan, 2004	Kenya, bucket samples	$10^3 - 10^5$

2.5.3 Microorganisms associated with disease in camel’s raw milk:

2.5.3.1 *Staphylococcus species:*

Staphylococci are small Gram-positive cocci belonging to the family of Micrococcaceae. The species can be subdivided into two groups showing either coagulase positive or coagulase negative reactions (Kloos and Schleifer, 1986).

The presence of *S. aureus* in camel milk is reported by El-Ziney and Al-Turki (2007) with a prevalence of 70 % in the milk samples of healthy camels in Saudi Arabia (Qassim Region) and 12% - 16.7% in Ethiopia (Abdurahman,2006 and Adugna *et al.*, 2013).

Table 3 : Staphylococci in camel milk

Author(s)	Country	<i>S.aureus</i> (%)	Samples	camels
Elhaj <i>et al.</i> , 2013	Sudan	28.69	160	160
Abdel Gadir <i>et al.</i> , 2005	Ethiopia	24.6	956	253
Abdurahman, 2006	Ethiopia	12	205	53
Adugna <i>et al.</i> , 2013	Ethiopia	16.7	24	24
Younan <i>et al.</i> , 2001	Kenya	11.0	1242	207
El-Jakee, 1998	Egypt	5.0	100	100
Barbour <i>et al.</i> , 1985	Saudi Arabia	17.1	205	205
El-Ziney and Al-Turki, 2007	Saudi Arabia (Qassim Region)	70	33	33
Wernery <i>et al.</i> , 2002	UAE	0.5	1313	14

2.5.3.2 *Escherichia coli*:

E. coli, a Gram-negative, non-sporulating facultative anaerobe, is an inhabitant of the intestines and faeces of warm-blooded animals and reptiles (Berg, 1996) and (Gordon and Cowling, 2003). *E. coli* is found in the gut microbiota, which consists of more than 500 species of bacteria that total 10^{10} – 10^{11} cells per gram of large-

intestinal content. Although the anaerobic bacteria in the bowel outnumber *E. coli* by 100/1 to 10,000/1 (Berg, 1996) *E. coli* is also known as pathogenic bacteria causing severe intestinal and extraintestinal diseases in man (Kaper *et al.*, 2004) as well as mastitis in camel (Bradley and Green, 2001).

Table 4 : *Escherichia coli* in camel milk

Author(s)	Country	<i>Escherichia coli</i> (%)	Samples	No. of camels
Elhaj <i>et al.</i> , 2013	Sudan	39.13	160	160
Abdel Gadir <i>et al.</i> , 2005	Ethiopia	17.3	956	253
Barbour <i>et al.</i> , 1985	Saudi Arabia	1.5	205	205
El-Jakee, 1998	Egypt	1.0	100	100

2.5.3.3 *Bacillus cereus*:

Bacillus cereus is a Gram-positive facultative anaerobic rod of the genus *Bacillus*. It is a widespread bacterium with the ability to form spores with high resistance against environmental influences. *B. cereus* is the cause of two different types of foodborne disease in man: a diarrhoeal type due to the production of enterotoxins in the small intestine and an emetic type, which is caused by the ingestion of a toxin (cereulide) produced in the foodstuff (Wegschneider, 2004;

Becker *et al.*, 2005; EFSA, 2003). The presence of *B. cereus* in camel milk is reported by Saad and Thabet (1993) with a prevalence of 29.4 % in the milk samples of healthy camels in Egypt. Abdel Gadir *et al.* (2005) proved the presence of *B. cereus* in 9.1 % of 956 quarter milk samples taken from 253 traditionally managed lactating camels in Ethiopia. Albrecht (2003) reported the presence of *B. cereus* in the sand of a camel dairy farm in the UAE.

2.5.3.4 *Salmonella* species:

Salmonella spp. are Gram-negative, facultative anaerobic rods with more than 2500 known serovars that belong to the family Enterobacteriaceae. *Salmonella spp.* are of high importance in food safety being able to provoke severe intestinal infections in humans which can lead to death especially in elderly people (Kleer, 2004; WHO, 2005). As in most animals, *salmonella* infections are common in camels in countries all over the world. Whereas some of the affected animals show clinical symptoms; others do not (Fazil and Hofman, 1981; Wernery, 2000; Semereab and Molla, 2001).

Burgemeister (1974) proved the presence of a serological reaction to *Salmonella Typhimurium* and *S. enteritidis* antigens each in 5.8 % of the examined camels. The presence of *Salmonella spp.* in camel milk is reported by El-Ziney and Al-Turki (2007) with a prevalence of 24 % in the milk samples of healthy camels in Saudi Arabia (Qassim Region). No cases of lactogenic transmission from camels to humans have yet been reported Younan (2004). The most frequent reason for the presence of *Salmonella spp.* in milk is through faecal contamination after heat treatment as salmonellae are inactivated during pasteurization (Kleer, 2004).

2.5.3.5 *Streptococcus* species:

The presence of *Streptococcus spp.* is mentioned in most articles in connection with the hygiene of camel milk. When a differentiation was done, mainly *Streptococcus agalactiae*, *S. dysgalactiae* and *S. uberis* were found in camel milk (Almaw and Molla, 2000; Younan, 2004).

Streptococcus agalactiae

Streptococcus agalactiae or Group B Streptococcus (GBS) are spherical cell shaped, non-motile, chain-forming and nonspore-forming, Gram-positive bacteria. In Gram-positive bacteria the cell wall is composed predominantly of peptidoglycan on which various carbohydrates, bacterial polysaccharides (teichoic acid) and surface antigens are attached. The cell wall polysaccharides of streptococcal species are critically important in determining the Lancefield serological grouping of strains on the basis of surface protein antigen (Lancefield, 1933). Capsular polysaccharide antigen and surface protein antigen determined ten serotypes Ia, Ib and II to IX in Group B Streptococcus. Majority of the neonatal infections in humans are caused by types I, II, III, and V (Whiley and Hardie, 2009; Imperi *et al.*, 2010).

Table 5 : *Streptococcus spp.* in camel milk

Author(s)	Country	Streptococcus spp. (%)	<i>S. agalactiae</i>	Samples	camels
Elhaj <i>et al.</i> , 2013	Sudan	05.21	–	160	160
Abdel Gadir	Ethiopia	7.0*	2.6	956	253

<i>et al.</i> , 2005					
El-Jakee, 1998	Egypt	12.0**	8.0	100	100
Barbour <i>et al.</i> , 1985	Saudi Arabia	4.9	–	205	205
Tuteja <i>et al.</i> , 2003	India	20.9	10.4	282	71

* All isolates 7% were *S. uberis* .

** 4% were *S. uberis* and rest 8% were unidentified *Streptococcus spp.*

2.5.3.6 *Listeria species:*

Listeria are Gram-positive widespread rods with a high resistance against environmental influences as cold, drought and solar radiation and are growing well in cold environment Terplan *et al.* (1986). Of the *Listeria* genus, mainly *Listeria monocytogenes* is of health importance for animals and humans, whereas other species as *L. ivanovii* and *L. seeligeri* are of minor importance in this respect or are considered as non pathogenic as *L. innocua*. The most common symptoms of listeriosis caused by *L. monocytogenes* are the dysfunction of the central nervous system, abortion and diarrhoea with possible lethal outcome, especially in predisposed individuals like pregnant women, children, elderly and immunosuppressed people. Very few is reported about listeria infections in old world camels. According to Burgemeister *et al.* (1975), a serological positive reaction was observed in 34.6 % of the tested camels in Tunisia. And 4.1% in Eastern Ethiopia (Adugna *et al.*, 2013)

2.5.3.7 *Haemophilus* species:

Are Gram negative spherical, oval or rod-shaped cells less than 1µm in width, variable in length, with marked pleomorphism, and sometimes forming filaments. The optimum growth temperature is 35–37°C. They are facultatively anaerobic and non-motile. Members of the *Haemophilus* genus are typically cultured on blood agar plates as all species require at least one of the following blood factors for growth: haemin (factor X) and/or nicotinamide adenine dinucleotide (factor V). Chocolate agar is an excellent *Haemophilus* growth medium as it allows for increased accessibility to these factors. Alternatively, *Haemophilus* is sometimes cultured using the "Staph streak" technique: both

Staphylococcus and *Haemophilus* organisms are cultured together on a single blood agar plate. In this case, *Haemophilus* colonies will frequently grow in small "satellite" colonies around the larger Staphylococcus colonies because the metabolism of Staphylococcus produces the necessary blood factor by-products required for *Haemophilus* growth. All *Haemophilus* species grow more readily in an atmosphere enriched with CO₂; *H. ducreyi* and some nontypable *H. influenzae* strains will not form visible colonies on culture plates unless grown in CO₂-enriched atmosphere. *Aggregatibacter aphrophilus* and *Haemophilus paraphrohaemolyticus* require CO₂ for primary isolation. On chocolate blood agar, colonies are small and grey, round, convex, which may be iridescent, and these develop in 24 hours. Iridescence is seen with capsulated strains. Carbohydrates are catabolised with the production of acid. A few species produce gas. Nitrates are reduced to nitrites (Ledebøer, 2011).

2.5.3.8 *Pseudomonas* species:

Pseudomonas species are aerobic, non-spore forming, Gram negative rods which are straight or slightly curved and are 0.5 – 1.0µm by 1.5 – 5.0µm. They are

motile by means of one or more polar flagella. They have a very strict aerobic respiratory metabolism with oxygen but in some cases, nitrate has been used as an alternative that allows anaerobic growth. Most species are oxidase positive (except *Ps. luteola* and *Ps. oryzihabitans*) and catalase positive. Other characteristics that tend to be associated with *Pseudomonas* species (with some exceptions) include secretion of pyoverdine, a fluorescent yellow-green siderophore under iron-limiting conditions. Certain *Pseudomonas* species may also produce additional types of siderophore, such as pyocyanin by *Pseudomonas aeruginosa* and thioquinolobactin by *Pseudomonas fluorescens*⁶. They grow well on standard broth and solid media such as blood agar, chocolate agar, and MacConkey's agar, which are recommended to isolate *Pseudomonas* species from clinical specimens. Selective agar containing inhibitors such as cefrimide can also be used for isolation and presumptive identification. *Pseudomonas* colonies may be nearly colourless, but white, off-white, cream, and yellow colony pigmentation is common. Fluorescent colonies can be readily observed under ultraviolet light. The type species is *Pseudomonas aeruginosa* (Henry D, 2011).

Pseudomonas aeruginosa

P. aeruginosa is the glucose non-fermenting Gram negative rod most often associated with human infection. It has the characteristic grape-like smell of aminoacetophenone. It is a strict aerobe with a growth temperature range of 35-42°C. Most other pseudomonads will not grow at 42°C (with certain exceptions, notably *Burkholderia pseudomallei*). The characteristic blue-green appearance of colonies/infected pus or of an organism culture is due to the mixture of pyocyanin (blue) and pyoverdine (fluorescein, yellow). Production of blue-green pigment is indicative of *Ps. aeruginosa*¹. Some strains produce other pigments, such as pyorubin (red) or pyomelanin (brown). Almost all strains are motile by means of a single polar flagellum. *Ps. aeruginosa* can produce at least six colonial types after

aerobic incubation on nutrient agar for 24hr at 37°C. The most common, type 1, is that of colonies which are large, low, oval, convex and rough, sometimes surrounded by serrated growth. Colonial variation from one type to another does not necessarily indicate the presence of more than one strain. Many strains exhibit metallic iridescence with colonial lysis. This resembles lysis by bacteriophage, but is not associated with phage activity. Colonies isolated on Pseudomonas selective or blood agar may be presumptively identified by a positive oxidase reaction and characteristic pigment production as '*Ps. aeruginosa*'. However, some strains of

Ps. aeruginosa, particularly the mucoid ones, may not produce pyocyanin, as well as displaying a slow oxidase reaction and may therefore require further tests to confirm identification. Colonies isolated on other selective agars (such as Bcc) may be identified by colonial morphology and a commercial identification system. Other species from blood or selective media and strains of *Ps. aeruginosa* and *B. cepacia* complex requiring further characterisation should be identified by a commercial identification system and/or referral to a Reference Laboratory. It should be noted that isolates from cystic fibrosis patients can be atypical/stressed and should be incubated at 30°C or room temperature for 48hrs so that their phenotypic features may reliably be expressed (Pitt, 1980).

Pseudomonas aeruginosa was isolated by four of seven authors from healthy camels with a prevalence of 1.0 - 17.7 %. No isolation of samples from camel udders with clinical mastitis is reported. In Khartoum (01.73%) from isolates contain *Pseudomonas spp.* (Elhaj *et al.*, 2013), In Eastern Ethiopia was (2%) reported by (Adugna *et al.*, 2013).

2.5.3.9 *Micrococcus species*:

Micrococcus species are strictly aerobic Gram positive cocci arranged in tetrads or irregular clusters, not in chains and cells range from 0.5 to 3µm in diameter. They are seldom motile and are non-sporing. They are also catalase positive and often oxidase positive, although weakly. Micrococci may be distinguished from staphylococci by a modified oxidase test (Baker Js, 1984) and (Faller, 1981). Their colonies are usually pigmented in shades of yellow or red and grow on simple media. The optimum growth temperature is 25-37°C. They have a respiratory metabolism, often producing little or no acid from carbohydrates and are usually halotolerant, growing in 5% NaCl. They contain cytochromes and are resistant to lysostaphin (Holt, 1994). They are generally considered harmless saprophytes that inhabit or contaminate the skin, mucosa, and also the oropharynx; however they can be opportunistic pathogens in certain immunocompromised patients (Kloos, 1999). There are currently 9 species of *Micrococcus* and 2 have been known to cause infections in humans - *Micrococcus lylae* and *Micrococcus luteus* (Euzeby, 2013).

2.5.3.10 *Aerococcus species*:

There are seven species of *Aerococcus*, of which five are pathogenic and cause both urinary tract and invasive infections (including Infective Endocarditis) in humans (Zhang *et al.*, 2000 and Lawson, 2001). They are *Aerococcus christensenii*, *Aerococcus sanguinicola*, *Aerococcus urinae*, *Aerococcus urinaehominis* and *Aerococcus viridans*. Aerococci resemble “viridans” streptococci on culture but differ microscopically by characteristically occurring as pairs, tetrads or clusters, similar to staphylococci. Sometimes a weak catalase or pseudocatalase reaction is produced. These relatively slow-growing organisms produce small, well-delineated, translucent, alpha-haemolytic colonies on blood agar. Some strains of *Aerococcus viridans* are bile aesculin positive and PYR

positive. *Aerococcus urinae* is bile aesculin negative and PYR negative. Growth occurs both under aerobic and anaerobic conditions. In some commercial identification systems, *Helcococcus kunzii* may be mis-identified as *A. viridans*. Both the API and Vitek also misidentify *A. sanguinicola* as *A. viridans*. This makes the reports of infections caused by *A. viridans* problematic when identification is based on these methods (Rasmussen, 2013). Most aerococci are sensitive to beta-lactams as well as to several other groups of antibiotics. *Aerococcus species* are sensitive to vancomycin although elevated MICs have been reported (Rasmussen, 2013).

2.5.3.11 *Corynebacterium species*:

Are Gram positive non-motile rods, often with clubbed ends, occurring singly or in pairs. Some cells may stain unevenly giving a beaded appearance. Their size is between 2-6µm in length and 0.5µm in diameter. They group together in a characteristic way, which has been described as the form of a "V", "palisades", or "Chinese letters". Metachromatic granules are usually present representing stored phosphate regions. They are aerobic or facultatively anaerobic and exhibit a fermentative metabolism (carbohydrates to lactic acid) under certain conditions. They are fastidious organisms, growing slowly even on enriched medium. Agar containing blood and potassium tellurite, such as Hoyle's tellurite medium, serves as a selective and differential medium. On blood agar, they form small greyish colonies with a granular appearance, mostly translucent, but with opaque centres, convex, with continuous borders. Their optimum growth temperature is 37°C. *C. diphtheriae* grows as pinpoint grey/black colonies on Hoyle's tellurite agar in 16-18hr and produces characteristic colonies after 48hr. Isolates of potentially toxigenic *Corynebacterium species* will also grow on blood agar. Colonial morphology varies among the species. *C. ulcerans* and *C. pseudotuberculosis*

colonies may be slightly β -haemolytic on blood agar. *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* are facultatively anaerobic, non-sporing, non-capsulated and non-acid-fast. These organisms are non-motile and catalase positive. *C. ulcerans* and *C. pseudotuberculosis* are both urease positive which may be used to distinguish them presumptively from *C. diphtheriae*. Strains of these species can all harbour the phage borne diphtheria toxin gene, which is required for the production of toxin (Ryan, 2004). Toxigenic strains may cause diphtheria or diphtheria-like illness. Possible toxigenic strains of *Corynebacterium species* should be referred to the Reference Laboratory for detection of toxin production as soon as possible. Non toxigenic strains of corynebacteria eg *C. ulcerans*, *C. jeikeium*, *C. striatum* and non-toxigenic *C. diphtheriae* are also known to cause infections in humans including pulmonary infection, leukaemia and endocarditis. Both *C. jeikeium* and *C. striatum* are non-haemolytic, urease negative and catalase positive (Coyle *et al.*, 1990).

2.5.3.12 *Rothia species*:

Rothia species are Gram positive cocci with a variable microscopic morphology. Their cells occur singly, in pairs, in clusters or in chains. They are weakly catalase positive and weakly proteolytic. *Rothia species* are positive for nitrate and nitrite reduction, liquefaction of gelatin and fermentation of sugars with the production of acid; while negative for motility, urease and indole. Colonies on agar surface may appear branched which rapidly fragment into bacillary or coccoid forms, resembling Actinomyces or *Nocardia species* (Georg, 1967). They exhibit good growth under aerobic or microaerophilic conditions, but poor or no growth anaerobically. *Rothia species* are susceptible to penicillin but because rare isolates may be resistant, susceptibility testing should be performed. There are currently 7

species of *Rothia* and 2 have been known to cause infections in humans - *Rothia dentocariosa* and *Rothia mucilaginoso* (Euzeby, 2013).

Rothia dentocariosa

R. dentocariosa cells occur singly, in pairs, in clusters or in chains. Colonial pleomorphism can also be observed. Microscopically, the morphology varies from coccoid to diphtheroid (with clavate ends) to filamentous. In broth cultures, cells may be coccoid, which distinguishes them from *Actinomyces* species and appears in filamentous forms on plates, but mixtures may appear in any culture (Von, 2004). They may show rudimentary branching and loss of the Gram positive appearance in ageing cultures. *R. dentocariosa* grows faster under aerobic than under anaerobic conditions, and does not need CO₂ or lipids for growth. It grows well on simple media (except Sabouraud dextrose agar) and colonies may be creamy, dry, crumbly or mucoid, nonhaemolytic and may adhere to the agar surface. They are non-motile, catalase positive and ferment carbohydrates with the end-products being lactic and acetic acid (Funke, 1997). Catalase negative strains of *R. dentocariosa* have been reported and this will be more difficult to recognise with traditional tests, since they may mimic the rare *Bifidobacterium* strains that are able to grow aerobically, as well as *Actinomyces* and *Arcanobacterium* species, *Propionibacterium propionicum* and catalase negative *Listeria* strains (Von, 2004). *R. dentocariosa* is distinct from *Dermabacter* species in that it is nitrate and pyrazinamidase positive.

The prevalence of *Pasteurella haemolytica* is given with 1.5 - 6.0 % by six out of seven authors in the milk of clinically inapparent camels and with 3.0 % by one author El-Jakee (1998) in samples of camels with clinical signs of mastitis.

Hafez *et al.*, (1987) did not specify the subspecies of the 16.7 % *Pasteurella spp.* isolated from 62 mastitis cases.

Chapter Three

MATERIALS AND METHODS

3.1 Description of the study area:

The study was carried out in Khartoum State, the capital of Sudan, which lies at the junction of the two rivers, the White and the Blue Niles in the North Eastern part of central Sudan. It lies between latitude 15°-16° N and longitude 21°-24° East with a length of 250 km and a total area of 20,736 km². The surface elevation ranges between 380 to 400 m above sea level (a.s.l.).

The state has an area of 5.2 million feddans (1 feddan = 0.42 ha²). Khartoum State is boarded by the River Nile State in the North, Gezeira State in the South, Elgadarif State in the East and North Kordofan in the West.

The main water source is the River Nile, white and Blue Nile and its tributaries, seasonal water source (wedians), and ground water – mainly in areas away from River Nile. In Khartoum State the occupation of non-governmental employees is in agriculture and animal husbandry since ancient times. Many individuals of these sectors who live in the outskirts of Khartoum town have become specialized in the breeding of cattle, camel, goat and sheep for milk and meat production.

The State is composed of seven localities namely Khartoum, Khartoum North, East Nile, Omdurman, Karary, Ombda and Jabelawlia. Most of Khartoum State falls within the semi-arid climatic zone while the Northern part of it falls within the arid climatic zone. The state is prevailed with a hot to very hot climate, and with rainy season during the summer and warm to cold dry during winter. The rainfall ranges between 100-200 mm at the North Eastern parts to 200-300 mm at the Southern parts with 10-100 mm at the North Western parts. The temperature in summer ranges between 25-40 °C during the months of April-June and between 20-35 °C during July-October Period. The degree of temperature falls during the winter period between November-March to the level of 15-25 °C. Khartoum state is divided into three large towns, built at the convergence of the Blue and White Niles: Namely Omdurman to the northwest across the White Nile, Khartoum North, and Khartoum itself on the southern bank of the Blue Nile (Adel And Omer, 1999).

The relative humidity fluctuates during the day (GMT) and during the year (season). The mean annual relative humidity ranges between 26-21 % (Jan to Feb), 15-26 % (March to June) and 41-48 % (July to September, the wettest three months) (Van Der Kevie, 1973).

3.2 Raw milk sample collection:

Between May and June 2015, a total of eighty bulks camel milk samples (\approx 25 ml each) were collected from Three different locations in Khartoum State Shambat (Khartoum-North), Green Valley (East Nile) and West Soba (Khartoum). 40 milk samples (20 samples from West Soba and 10 samples from each of the other two farms) collected from camels by hand milking after disinfected the udder with 70% ethanol using a hand sprayer and dried with disposable towel. 40 milk samples from milk utensils (20 samples from Soba West and 10 samples from each of the other two farms). The camel milk samples were collected aseptically using sterile bottles and transported to Central Veterinary Research Laboratory (CVRL) Department of Bacteriology, Soba Khartoum by placing them in an icebox. The milk samples were kept at 4°C in a refrigerator until laboratory analysis.

Table 6 : Localities and number of milk samples

Localites	Number of samples	Number of camels
Khartoum-North	20	10
East Nile	20	10
Khartoum	40	20
Total	80	40

3.3 Microbiological assessment:

3.3.1 Equipment:

The equipment used for the tests were glass slides and cover slips, pipettes 0.01 ml, one ml, five ml and ten ml, bottles, syringes, test tubes, culture dishes,

spoons and loops, pastire loops, injection or droper, Durham tubes, clean 250 ml beakers, balance, incubator, 35 + 1° C. Water baths, 37+0.5° C, autoclave, 15 pounds pressure at 121° C and oven 160° C.

3.3.2 Sterilization:

Sterilization was done according to Barrow and Feltham (2003). The glass wares such as Petri dishes, test tubes, flasks and pipettes were sterilized using dry heat oven regulated at 160°C for 1 hours. The media, automatic pipette tips and distilled water were sterilized using steam autoclave at 121°C for 15 minutes (15 lb/ inch²).

3.3.3 Preparation of media:

All media were obtained in dehydrated form and were prepared according to the manufacturer's instructions. After taking appropriate weights, the stated distilled water was added to the media, then it was heated to dissolve completely and sterilized. Then cooled to 44 - 50°C. The media were poured aseptically (10-12 ml) into sterile plates by gentle lifting of the cover of the plate high enough to pour the medium. The plates were then allowed to solidify within five to ten minutes and inverted (Marshall, 1992).

3.3.4 Isolation and identification:

She-camel milk samples were kept at room temperature for five to ten minutes and a loopfull was inoculated by streaking onto 10% defibrinated sheep blood agar, MacConkey agar and nutrient agar. The plates were incubated aerobically at 37°C for 24-48 hours for bacterial growth. The plates were examined daily by the naked eye for growth and colonial morphology and characteristics such as shape, size, consistency, haemolysis or pigment production. Smears were made from primary

cultures, dried in the air, fixed by heating, stained by Gram's staining method and examined microscopically. Isolated bacterial colonies were purified by subculturing on nutrient agar and for suspected β -haemolytic streptococci on blood agar. Identification of the bacterial isolates was done according to the method outlined by Barrow and Feltham (2003).

The isolates were stored in a refrigerator and subcultured weekly on fresh sheep blood agar plates or nutrient agar and transferred to fresh medium when bacteriological studies were started.

3.3.4.1 Primary tests:

3.3.4.1.1 Morphological appearance:

The bacterial appearance and morphology were recorded according to Barrow and Feltham (2003).

3.3.4.1.2 Gram stain:

Gram stain technique was done as indicated by Harrigan and Mccance (1976). The tested organism was picked up using sterile wire loop and put in the drop of sterile normal saline, which was previously put in a sterile slide. Emulsified, spread, allowed to dry and then fixed by passing the smear three times over a flame. Crystal violet was added to the smear for one minute then washed with distilled water. Lugol's iodine was added for one minute and washed using distilled water. The slide was decolorized by ethanol for 10 seconds. The smear was then stained using saffranin for 20-30 seconds and rinsed using distilled water. The slides were dried by filter paper and one drop of immersion oil was added to the

slide for examining under the microscope. Gram positive organisms appeared purple, while Gram negative ones appeared pink.

3.3.4.1.3 Catalase test:

On a clean microscope slide, a loopful of 3% hydrogen peroxide was placed. A colony of test culture, on nutrient agar, was picked using a wooden stick or glass rod and put in the reagent. Production of gas bubbles indicated positive result.

3.3.4.1.4 Oxidase test:

Pieces of filter paper were soaked in 1% solution of tetramethylene p-phenylene diamine dihydrochloride and dried. A colony of an overnight growth, on nutrient agar, was picked with sterile bend glass rod and rubbed on the filter surface placed in a Petri dish. Development of dark violet colour within 60 seconds indicated positive test (Barrow and Feltham, 2003).

3.3.4.1.5 Motility test:

Young broth cultures of the organism, incubated at or below the optimum growth temperature (e.g. 37 °C and 22 °C), were examined in 'hanging drop' preparations, using a high-power dry objective and reduced illumination. Motile organisms was indicated by their movement in different directions (Barrow and Feltham, 2003).

3.3.4.1.6 Sugar fermentation test:

The medium was prepared by adding 1% of the required sugar to peptone water with Andred's indicator in a Bigu vials with inverted Durham tube and inoculated with tested organism and incubated at 37 °C and examined daily. Acid production was indicated by appearance of pinkish color, while gas production was indicated by presence of empty space in the inverted Durham's tube (Barrow and Feltham, 2003).

3.3.4.1.7 Oxidation Fermentation (O/F) test:

Two tubes of Hugh and Leifson's medium were inoculated with the test culture. One of the tubes was covered by a layer of sterile paraffin oil to about 3 cm above the surface of the medium, the other was left unsealed. Both were then incubated at 37°C and examined daily up to two weeks (Hugh and Leifson, 1953). The result read as follows:

- 1- Fermentative if both tubes were changed to pink color.
- 2- Oxidative if tube without oil was changed to pink color.
- 3- Negative result was indicated by no color changes in both tubes.

3.3.4.1.8 Carbon dioxide requirement:

An incubator in which the concentration of CO₂ can be regulated or an anaerobic jar from which the appropriate amount of air is evacuated and replaced with CO₂ are necessary for defined conditions; if these are not essential, a candle jar can be used which gives an atmosphere of about 2.5% CO₂ and 17% O₂ (Morton, 1945); for anaerobes, a CO₂ gas-generating kit in an anaerobic jar yields a final atmosphere of CO₂ (10%) and hydrogen in the absence of oxygen.

3.3.4.2 Secondary Biochemical tests:

3.3.4.2.1 Tube coagulase test:

To 0.5 ml of 1/10 dilution of plasma in saline add 0.1 ml of an 18-24-hour broth culture of the organism was added incubated at 37 °C and examined after 1, 3 and 6 h for coagulation. If negative, the tubes were left at room temperature overnight and then re-examined (Gillespie, 1943).

3.3.4.2.2 Methyl red (MR) reaction:

Glucose Phosphate (MR) medium was inoculated by the test organism and incubated at 30 °C for 5 days. Add 2 drops of methyl red solution were added, shaken and examined. A positive MR reaction was shown by the appearance of a

red colour at the surface. An orange or yellow colour should be regarded as negative (Barrow and Feltham, 2003).

3.3.4.2.3 Voges-Proskaur (V.P) test:

Glucose phosphate medium (M.R-V.P medium) was inoculated with the tested organisms and incubated at 37°C for 48 hours. An amount of 0.2 ml of 40% potassium hydroxide and 0.6 ml of 5% α -nephthal solution were added to one ml of culture, then shaken, placed in slope position and examined after 15 minutes and one hour, a positive reaction was indicated by bright pink or red colour (Barritt, 1936).

3.3.4.2.4 Indole production test:

Peptone Water or Nutrient Broth was inoculated with the test organism and incubated for 48 h. 0.5 ml Kovacs' reagent was added, shaken well, and examined after about 1 min. A red colour in the reagent layer indicates indole production (Barrow and Feltham, 2003).

3.3.4.2.5 Nitrate reduction:

Nitrate Broth was lightly inoculated with the test organism and incubated for up to 5 days. 1 ml of nitrite reagent A followed by 1 ml of reagent B were added. A deep red colour which shows the presence of nitrite and thus shows that nitrate has been reduced, indicated a positive reaction (Barrow and Feltham, 2003).

3.3.4.2.6 Phenylalanine deamination:

Malonate-phenylalanine medium was lightly inoculated and incubated for 18-24 h and 0.1 N-HCl was added drop by drop until the medium was yellow. Then 0.2 ml of a 10% aqueous solution of $FeCl_3$ was added; shaken and any colour change was observed immediately; a positive reaction was indicated by a dark green colour which quickly fades (Shaw and Clarke, 1955).

3.3.4.2.7 Urease activity:

A slope of Christensen's Urea medium was heavily inoculated with the test organism and examined after incubation for 4 hours and daily for 5 days. Red colour indicated positive reaction (Barrow and Feltham, 2003).

3.3.4.2.8 Citrate test:

A slope of Simmons' citrate medium was inoculated as a single streak over the surface. Examined daily for up to 7 days for growth and colour change. Positive results were confirmed by subculture to Simmons' or Koser's Citrate medium. blue colour and streak of growth indicated citrate utilization
original green colour indicated citrate not utilization (Barrow and Feltham, 2003).

3.3.4.2.9 CAMP test:

β -haemolytic *Staphylococcus aureus* was streaked in the middle of the surface of 5% blood agar plate. The organism under test was streaked vertically to that line and the plate was incubated over night at 37°C. Positive reaction was indicated by a half-moon shaped clear haemolysis when the line of the isolate passed the haemolytic zone of the *Staphylococcus aureus*.

3.3.4.2.10 Novobiocin sensitivity test:

A volume of two ml of diluted culture were spread on the surface of nutrient agar. The excess fluid was discarded and the plate was allowed to dry, then Oxoid discs of novobiocin (5 mg) were applied to the surface of the medium by sterile forceps and incubated at 37°C for 24 hours. Zone of inhibition was determined whether the organism was sensitive or not to novobiocin.

3.3.4.2.11 KCN test:

1 ml KCN Broth was inoculated with one loopful of an overnight broth culture or a light suspension of the organism. Cap of the bottle was screwed tight and incubated for up to 48 h and examined after 24 and 48 h for turbidity indicating growth, which constitute a positive reaction (Barrow and Feltham, 2003).

Controls: positive - *Klebsiella pneumoniae* subsp. *aerogenes*

negative - *Escherichia coli*

3.3.4.2.12 Arginine hydrolysis:

5 ml of Arginine Broth were inoculated and incubated for 24 h and 0.25 ml Nessler's reagent were added. Arginine hydrolysis was indicated by the development of a brown colour. For streptococci, 0.5 ml of culture was added to

4.5 ml distilled water, shaken and 0.25 ml Nessler's reagent added (Barrow and Feltham, 2003).

Controls: positive - *Enterococcus faecalis* negative - *Streptococcus salivarius*

3.4 Total viable bacterial count:

3.4.1 Preparation of serial dilution

The milk sample is serially diluted by adding 1x of suspension to 9x of diluents, dilutions were made to 10^{-8} . Three plates were needed for each dilution series.

3.4.2 Culturing method

The surface of the plates need to be sufficiently dry to allow a 20 μ l drop to be absorbed in 15–20 minutes. Plates were divided into equal sectors (up to 8 per plate). The sectors are labelled with the dilutions. In each sector, 1 x 20 μ l of the appropriate dilution was dropped onto the surface of the agar and the drop allowed to spread naturally (avoiding touching the surface of the agar with the pipette).

3.4.3 Incubation of the cultures

The plates were left upright on the bench to dry before inversion and incubation at 37 °C for 18 – 24 hours.

3.4.4 Counting of colonies

Each sector was observed for growth, high concentrations gave confluent growth over the area of the drop, or a large number of small/merged colonies. Colonies were counted in the sector where the highest number of full-size discrete colonies were seen (usually sectors containing between 2-20 colonies are counted). The following equation is used to calculate the number of colony forming units (CFU) per ml from the original aliquot / sample: CFU per ml = Average number of colonies for a dilution x 50 x dilution factor (Miles and Misra, 1938).

3.5 Media used:

3.5.1 Nutrient broth (Oxoid, CM1) (g/L):

Contents:

Lab-lemco powder	1.0
Yeast extract (Oxoid L 20)	2.0
Peptone (Oxoid L 37)	5.0
Sodium chloride	5.0

pH 7.4 (approx.)

Procedure:

Thirteen grams of the dehydrated powder were added to one litre of distilled water, mixed well and distributed into bottles in 5 ml amounts and sterilized by

autoclaving at 15 pressure per square inch (p.s.i) for 15 minutes. The prepared medium was kept at 4°C until used.

3.5.2 Nutrient agar (OXOID CM3) (g/L):

Contents:

Lab-lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar No 3	15

pH 7.4 (approx.)

Procedure: Twenty eight grams were suspended in one litre of distilled water and brought to the boil to dissolve completely, then sterilized by autoclaving at 121°C for 15 minutes, cooled to 45-50°C and distributed into sterile Petri dishes in 15 ml portion each. The medium was kept at 4°C until used.

3.5.3 Peptone water (OXOID CM1049) (g/L):

Contents:

Peptone	10.0
Sodium chloride	5.0

pH 7.4 (approx.)

Procedure: Fifteen grams were added to one litre of distilled water, mixed well and distributed into ten ml test tubes in three ml amounts, and sterilized by

autoclaving at 121°C for 15 minutes. The prepared medium was kept at 4°C until used.

3.5.4 Blood agar base (OXOID CM0055) (g/L):

Contents:

Nutrient agar 900 ml

Sterile defibrinated blood 100 ml

Procedure:

The nutrient agar was prepared according to the manufacturer's instructions, sterilized by autoclaving at 121°C for 15 minutes, cooled to 50°C and aseptically sterile blood was added and thoroughly mixed. Formation of air bubbles was avoided. The blood was allowed to warm to room temperature before being added to the molten agar, dispensed aseptically in 15 ml amounts in sterile Petri dishes. Each batch of the medium was labeled by a number and date. The plates were then stored at 4°C in sealed plastic bags to prevent loss of moisture. Depending upon the agar base used, the pH was within the range of 7.2-7.6 at room temperature. The prepared medium was kept at 4°C until used.

3.5.5 MacConkey's agar (OXOID, CM7b) (g/L):

Contents:

Peptone 20

Lactose 10

Bile salts 5

Sodium chloride 5

Neutral red 0.075

Agar No 3

12

pH 7.4 (approx.)

Procedure: Fifty two grams were suspended in one litre of distilled water, boiled until dissolved completely and sterilized by autoclaving at 121°C for 15 minutes, then poured into sterile Petri dishes in portions of 15 ml and then stored at 4°C until used.

3.5.6 Hugh and Leifson's (O/F) medium (Barrow and Feltham, 1993):

Contents:

Peptone	2.0
Sodium chloride	5.0
Agar	3.0
K ₂ HPO ₄	0.3
Distilled water	1000 ml
Bromothymol blue, 0.2% eq. Sol	15 ml

Procedure:

The ingredients were dissolved by heating in water bath set at 55°C, the pH was adjusted to 7.1, then the indicator was added and the medium sterilized at 115C for 20 minutes. A volume of 10 ml of sterile glucose solution was aseptically added to 90 ml of medium. Then the medium was mixed and distributed aseptically in ten ml amounts into sterile test tubes. The prepared medium was kept at 4°C until used.

3.5.7 Motility medium (Barrow and Feltham, 1993) (g/L):

Contents:

Peptone	10.0
Meat extract	3.0

Sodium chloride 5.0
Agar 4.0
Gelatin 80 Distilled water 1000

Procedure:

The gelatin was soaked in water for 30 minutes, then the other ingredients were added, heated to dissolve, and sterilized at 115°C for 20 minutes. The prepared medium was kept at 4°C until used.

3.5.8 MR VP medium (OXOID CM43) (g/L):

Contents:

Peptone	5.0
Dextrose	5.0
Phosphate buffer	5.0
Distilled water	1000 ml

pH 7.5 (approx.)

Procedure:

Fifteen grams were added to one litre of distilled water, mixed well, then distributed into test tubes and sterilized by autoclaving at 121°C for 15 minutes. The prepared medium was kept at 4°C until used.

3.5.9 Simmon's citrate agar (OXOID CM0155) (g/L):

Contents:

Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	0.2
Sodium ammonium phosphate	0.8
Sodium citrate, tribasic	2.0
Sodium chloride	5.0

Bromothymol blue	0.05
Agar No 3	15
pH 7.0 (approx.)	

Procedure:

Twenty three grams were suspended in one litre of distilled water, boiled to dissolve completely and sterilized by autoclaving at 121°C for 15 minutes, then the medium was distributed in ten ml portions into sterile MacCarteny bottles aseptically and allowed to set in slope position. The prepared medium was kept at 4°C until used.

3.5.10 Agar 2.0 (OXOID CM49) (g/L):

Peptone water 1000 ml

Procedure:

The ingredients were dissolved in peptone water, sterilized by autoclaving at 115°C for 10 minutes, poured into sterile MacCarteny bottles aseptically and allowed to set in slope position to solidify, then stored at 4°C until used.

3.5.11 MacConkey broth (OXOID CM5) (g/L):

Content:

Peptone	20
Lactose	10
Bile salts	5.0
Sodium chloride	5.0

Neutral red 0.075
pH 7.4 (approx.)

Procedure:

Fourty grams was added to one litre of distilled water, mixed well, distributed into test tubes, fitted with Durham tube and autoclaved at 121°C for 15 minutes. The prepared medium was kept at 4°C until used.

Statistical analysis:

For data entry and analysis SPSS version 20 was used. Percentages were used to express the proportion of bacterial isolates. The difference in bacterial load between the milk samples from the udder and milking vessels was analyzed using Chi-Square test. The result was reported as significant if P-value was less than 5 %.

Chapter Four

Results

4.1 Bacterial isolates:

In the udder milk samples the presence of *Staphylococcus spp.* were isolates 27 (36%), *Staph aureus* 6 (8%), *Micrococcus spp.* 2 (2.6%), *Bacillus spp.* 1 (1.3%) and *Enterobacter spp.* 1 (1.3%) whereas the utensil's milk samples showed the presence of *staphylococcus spp.* isolates was 20 (26.6%), *Staph aureus* 3 (4%), *Acinetobacter spp.* 3 (4%), *Micrococcus spp.* 2 (2.6%), *Streptococcus spp.* 2 (2.6%), *Nocardia spp.* 2 (2.6%), *Bacillus spp.* 1 (1.3%), *Rothia dentocariosa* 1 (1.3%), *Neisseria spp.* 1 (1.3%) and *Aerococcus spp.* 1 (1.3%) (Table 7).

Table 7 : Different bacterial isolates from udder and utensil milk:

Bacterial isolates	Udder	Milking utensils	Total
<i>Staphylococcus spp.</i>	27 (72.9%)	20 (52.6%)	47(62.6%)
<i>Staph aureus</i>	6 (16.2%)	3 (7.8%)	9(12%)
<i>Acinetobacter spp.</i>	-	3 (7.8%)	3(4%)
<i>Haemophilus spp.</i>	-	2 (5.2%)	2(2.6%)
<i>Micrococcus spp.</i>	2 (5.4%)	2 (5.2%)	4(5.3%)
<i>Bacillus spp.</i>	1 (2.7%)	1 (2.6%)	2(2.6%)
<i>Streptococcus spp.</i>	-	2 (5.2%)	2(2.6%)
<i>Rothia dentocariosa</i>	-	1 (2.6%)	1 (1.3%)
<i>Enterobacter spp.</i>	1 (2.7%)	-	1 (1.3%)
<i>Neisseria spp.</i>	-	1 (2.6%)	1 (1.3%)
<i>Nocardia spp.</i>	-	2 (5.2%)	2(2.6%)
<i>Aerococcus spp.</i>	-	1 (2.6%)	1 (1.3%)
No bacterial growth	14	9	23
Total bacterial isolates	37	38	75

Table 8 : biochemical reactions from the isolated bacteria:

Tests	The isolated bacteria									
	<i>Staphylococcus spp.</i>	<i>Micrococcus spp.</i>	<i>Bacillus spp.</i>	<i>Acinetobacter spp.</i>	<i>Haemophilus spp.</i>	<i>Streptococcus spp.</i>	<i>Nocardia spp.</i>	<i>Enterobacter spp.</i>	<i>Neisseria spp.</i>	<i>Aerococcus spp.</i>
Gram stain	+	+	+	-	-	+	+	-	-	+
Shape	Cocci	Cocci	Rod	Cocci	Cocci/Rod	Cocci	Rod	Rod	Cocci	Cocci
Motility test	-	-	+	-	-	-	-	+	-	-
Growth in air	+	+	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	-	+	-	+	+
Oxidase test	-	+	-	-	-	-	+	-	+	-
Glucose	+	+	+	+	-	+	+	+	+	+

Carbohydrate breakdown	F	-	+	+	+	F	O	F	+	F
Citrate utilization	NA	NA	-	NA	NA	NA	NA	NA	NA	NA
Indole test	NA	NA	NA	NA	NA	NA	NA	-	NA	NA
Voges-Proskauer	+	NA	NA	NA	NA	-	NA	-	NA	-
Growth on MacConky	NA	NA	NA	NA	NA	NA	NA	+	NA	NA
Growth in 6.5% NaCl	+	NA	NA	NA	NA	NA	NA	+	NA	NA
Nitrate	+	NA	+	-	+	NA	NA	NA	-	+
Arginine	NA	NA	NA	NA	-	-	NA	-	NA	NA
Urease	+	NA	-	-	NA	NA	+	NA	NA	+
Methyl red	NA	NA	NA	NA	NA	NA	NA	-	NA	NA

NA = Not available

F = Fermentative

O = Oxidative

Table 9 : Biochemical reactions for *Staphylococcus aureus* :

Tests	Results
Gram stain	Positive
Shape	Cocci
Motility test	Negative
Growth in air	Positive
Catalase test	Positive
Oxidase test	Positive
Glucose	Positive

Carbohydrate breakdown	Fermentative
Voges-Proskauer	Positive
Nitrate	Positive
Arginine	Positive
Maltose	Positive
Mannitol	Positive
Fructose	Positive
Sucrose	Positive
Raffinose	Negative
Mannose	Positive

Table 10 : Biochemical reactions for *Rothia dentocariosa* :

Tests	Results
Gram stain	Positive
Shape	Rod
Motility test	Negative
Growth in air	Negative
Catalase test	Positive
Oxidase test	Positive
Glucose	Positive

Carbohydrate breakdown	Fermentative
Voges-Proskauer	Negative
Nitrate	Positive
Indole	Negative
Maltose	Positive
Mannitol	Negative
Sucrose	Positive
Raffinose	Negative

4.2 The total viable bacterial count:

The standard deviation of viable bacterial count (Table 9) was 6.9×10^5 cfu/ml in udder milk samples and 5.6×10^6 cfu/ml in utensil's milk samples. The difference between the viable bacterial count in udder and utensils milk samples, were statistically not significant.

Table 11 : Bacterial count of udder and utensils milk samples

Milk sample site	Bacterial count
-------------------------	------------------------

From udder	698,750 ± 883.883
From utensils	5,633333.0 ± 329982.69
Level of significant	NS 0.317

Values are means ± standard deviation.

NS = not significant

Chapter Five

Discussion

Major bacterial isolates from udder was *Staphylococcus spp.* and this agrees with Brihanu *et al.* (2008) also as from milking utensils *Staphylococcus spp.* represented the higher percentage. *Bacillus spp.* and *Micrococcus spp.* have same percent in udder and utensils milk samples. *Enterobacter spp.* was isolated only from udder milk.

In this study *Staphylococcus spp.* represented (62.6%) which agrees with El-Ziney and Al-Turki (2007) who found that *staph aureus* in raw camel milk

represented (70%). In Khartoum Elhaj *et al.* (2013) reported that the isolated *Stapylococcus spp.* from raw camel milk was (28.69%) and Omer and Eltinay (2008) who found that *Stapylococcus spp.* as (32%) in central and southern regions of United Arab Emirates. In Eastern Ethiopia Adugna *et al.* (2013) found *Stapylococcus spp.* as (12.7%).

In this study (4%) of the isolates were *Acinetobacter spp.* This is less than Adugna *et al.* (2013) who found that *Acinetobacter spp.* as (7.4%) In Eastern Ethiopia Brihanu *et al.* (2008) found (18.18%) as *Acinetobacter spp.* .

In this study (12%) of isolates were *Staph aureus*, this agrees with Abdurahman (2006) who found (12.7%) of camel milk in the Errer valley of eastern Ethiopia contain *Staph aureus*. While Brihanu *et al.* (2008) found *Staph aureus* as (7.14%) in Ethiopia. Isolation percentage of *Staph aureus* in this study is less than Eldemerdash (2012) in Egypt who found (30%) of samples contain *Staph aureus*, Adugna *et al.* (2013) who found (16.2%) of samples was *Staph aureus* in Eastern Ethiopia this, result approaches that of Younan (2001) in Kenya and Barbour *et al.*, (1985) in Saudi Arabia containing (17%) and (17.5)% respectively. El-Zainey and Al-Turky, (2007) in Saudi Arabia found high level of *Staph aureus* (70%).

Micrococcous spp. in this study as (5.3%) agreed with Barbour *et al.* (1985) who found 5% of isolates as *Micrococcous spp.* in Abu Dhabi, United Arab Emirates and Adugna *et al.*, (2013) who found (4.7%) as *Micrococcous spp.* in Eastern Ethiopia, but less than Elhaj *et al.* (2013) in Khartoum found that *Micrococcous spp.* (7.82%) while Abdurahman (2006) in Ethiopia found that *Micrococcous spp.* as low as (1.3%).

Streptococcus spp. in this study accounted as(2.6%) of the isolates, while disagrees with Elhaj *et al.* (2013) in Khartoum, Adugna *et al.* (2013) in Eastern Ethiopia and Abdurahman (2006) in Ethiopia who reported (5.21%), (13.5%) and (26%) respectively.

In this study *Bacillus spp.* represented (2.6%) which is higher than Brihanu *et al.* (2008) who found *Bacillus spp.* as (1.79%), but less than Adugna *et al.* (2013) found (5.4%) of isolates as *Bacillus spp.* in Eastern Ethiopia.

In this study *Enterobacter spp.* accounted (1.3%) which disagrees with Adugna *et al.* (2013) who found (14.9%) of isolates as *Enterobacter spp.* in Eastern Ethiopia.

In this study *Haemophilus spp.*, *Neisseria spp.* and *Rothia dentocariosa* were isolated as (2.6%), (1.3%) and (1.3%) respectively.

No *Escherichia coli*, *klebsiella spp.*, *Pseudomonas spp.*, and *proteus spp.* was isolated from the examined camel milk, which were reported in previous studies.

In the present work *Escherichia coli*, *Salmonella species*, *Listeria monocytogenus*, *Corynebacterium spp.* and *Clostridium perfringens* were not isolated from all samples of raw camel's milk which has a significant public health implication.

Staphylococcus aureus, *Escherichia coli*, *Pseudomonas spp.*, *Bacillus spp.*, and *Alcaligenes spp.* were reported as common microflora of raw milk (Sherikar *et al.*, 2004). *Staphylococcus aureus* (Bekele And Molla, 2001 and Pascal, 1994).

The isolation of species of *Streptococcus spp.* only from milking utensils samples may indicate that these were the common environmental contaminants.

The absence of growth of bacteria in 23 (23.4%) cultures was almost in agreement with previous reports (Pascal, 1994) maybe she-camel under antibiotic treatment.

In the present study the main representative species included *Stapylococcus species* (62.6%) that may be due to the bad hygiene in small camels' farms in Sudan, or due to un- clean worker's hands or dirty utensils.

The total viable bacterial count ranged between 6.9×10^5 cfu/ml to 5.6×10^6 cfu/ml, according to the (SSMO, 2007) and (U.S Department Of Health, 1953) ; most of samples were classified as good because the total bacterial count were less than 50×10^5 cfu/ml. It is worth to mention that there are no microbiological limit values standards for camel milk. There for the microbiological limits values for cow's milk were used to assess the quality of camel's milk.

The result showed that there are no significant variations between the total viable bacterial count of udder and utensils milk samples.

Udder milk TBC 6.9×10^5 cfu/ml was less than (Younan, 2004) who found that the TBC for camel's raw milk collected from udder directly in Kenya was 1×10^2 cfu/ml to 1×10^4 . But Milking utensils TBC agrees with Brihanu *et al.* (2008) in Ethiopia who found bacterial count in udder milk 3×10^5 cfu/ml and in milking utensils 50×10^5 cfu/ml.

Our study result disagree with El Tahir (2005) in Khartom who found that the total bacterial count of camel's milk ranged between 0.11×10^5 cfu/ml to 0.39×10^9 cfu/ml this was very high compared with our results.

Very low bacterial count was detected in U.A.E 9.2×10^2 cuf/ml (Valérie *et al.*, 2007). In another study in U.A.E bacterial count varied between 5×10^2 cfu/ml to

7.4×10^5 cfu/ml and that the TBC in 94.1% of raw camel's milk samples was less than 1×10^5 cfu/ml (Werenry *et al.*, 2002).

Conclusion

- Although the hygienic measures were not properly established in small milk producing units, the result of total viable count of bacteria ranged between 6.9×10^5 cfu/ml to 5.6×10^6 cfu/ml and most of samples were classified as good according to the tropical standards, U.S and SSMO.

- The statistical analysis showed that there was no significance variations between the total viable bacterial count of udder and utensils camel's raw milk.
- The predominant bacteria in camel's raw milk were *Staphylococcus spp.* (62.6%), *Staphylococcus aureus* (12%) and *Micrococcus spp.* (5.3%).
- Hand milking methods, poor farm management and practices were observed as human hazards in all farm studied if milk is consumed unpasteurized.

Recommendations

- Training and guidance programs should be started in order to develop awareness among farmers emphasizing the need for hygienic practice at farm level.
- Good management practices should be directed, such as cleaning, applying personal and equipment hygiene during milking process.
- The udder and teats should be washed and cleaned before milking.

- Transportation and storage of raw milk should be at low temperature in clean cold steel or plastic tank to avoid bacterial growth in raw milk.
- Utensils should be properly washed prior to transferring milk to them.
- The raw camel's milk must be pasteurized or heated before direct drinking.
- More research should be done to investigate risk factors involved in contamination of raw camel milk.

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